PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/29, 15/82, 15/11, 5/10, C07K 14/415, A01H 5/00 — A1

(11) International Publication Number: WO 97/23618

(43) International Publication Date: 3 July 1997 (03.07.97)

(21) International Application Number: PCT/GB96/03191

(22) International Filing Date: 20 December 1996 (20.12.96)

(30) Priority Data:
9526218.4 21 December 1995 (21.12.95) GB

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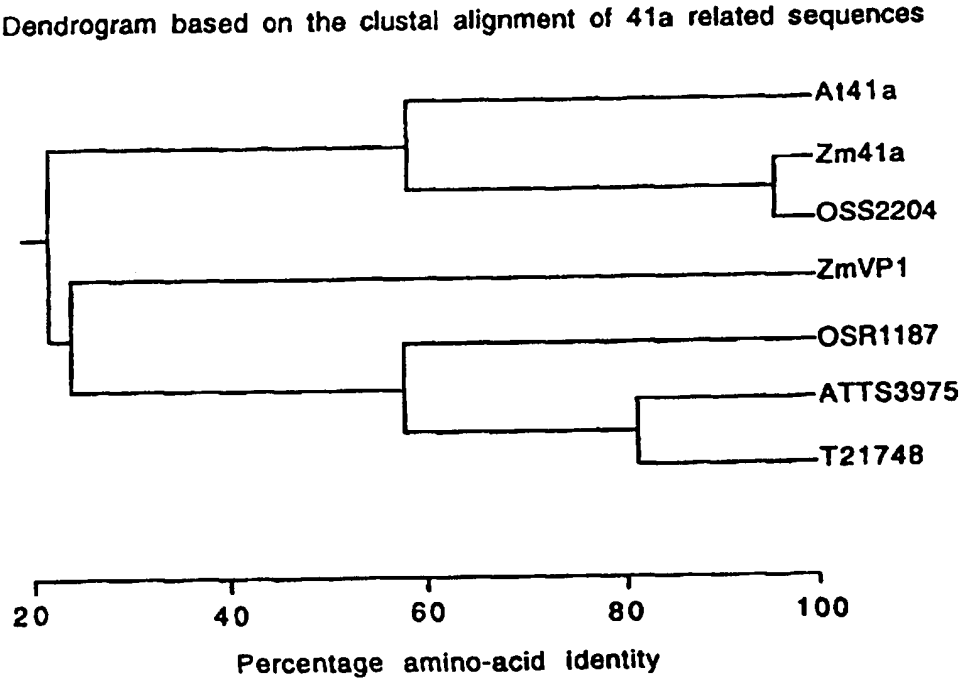
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA SEQUENCES CODING FOR A PROTEIN CONFERRING MALE STERILITY



(57) Abstract

Nucleic acid coding for a protein which confers male sterility is provided together with its use in producing transgenic plants.

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DNA SEQUENCES CODING FOR A PROTEIN CONFERRING MALE STERILITY

This invention relates to recombinant, isolated and other synthetic DNA useful in male-sterility systems for plants. In particular, the invention relates to a gene associated with male fertility, labelled Ms41-A, and a recessive mutant form thereof, labelled ms41-A, which confers male sterility. Male-sterile plants are useful for the production of hybrid plants by sexual hybridisation.

Hybrid plants have the advantages of higher yield and better disease resistance than their parents, because of heterosis or hybrid vigour. Crop uniformity is another advantage of hybrid plants when the parents are extensively homozygous; this leads to improved crop management. Hybrid seed is therefore commercially important and sells at a premium price.

Producing a hybrid plant entails ensuring that the female parent does not self-fertilise. There have been many prior proposals, mechanical, chemical and genetic, for preventing self-pollination. Among the genetic methods is the use of anther-specific genes or their promoters to disrupt the normal production of pollen grains. An anther-specific promoter, for example, can be used to drive a "male-sterility DNA" at the appropriate time and in the right place. Male sterility DNAs include those coding for lytic enzymes, including those that lyse proteins, nucleic acids and carbohydrates. Glucanases are enzymes which break down carbohydrates.

WO-A-9302197 describes recombinant or isolated DNA encoding a glucanase called callase.

Aarts *et al*, (*Nature*, **363**:715-717 (1993)) have described a gene required for male fertility, isolated from *Arabidopsis*, which has been labelled Ms2.

We have now identified and isolated from *Arabidopsis* another gene linked to male fertility. This gene has been labelled Ms41-A. Its mutant , recessive, form is labelled ms41-A and is capable of conferring male sterility. This gene would appear to offer advantages over Ms2 when used to produce male sterile plants.

Thus, in a first aspect the present invention provides recombinant or isolated Nucleic acid which:

a) encodes the Ms41-A protein from *Arabidopsis*;

b) encodes a Ms41-A like protein;

c) encodes the ms41-A protein from *Arabidopsis*;

d) encodes a ms41-A like protein;

e) comprises a promoter sequence which regulates expression of the Ms41-A protein from *Arabidopsis* or a promoter sequence which regulates expression of a Ms41-A like protein; or

f) hybridises under stringent conditions to Nucleic acid a), b), c), d) or e) or would do so but for the degeneracy of the genetic code.

In one embodiment of a) above, the Nucleic acid encodes a protein having an amino acid squence as shown in figure 4. Although figure 4 relates only to a protein of

Arabidopsis, those skilled in the art will readily be able to identify equivalent proteins from other members of the family *Brassicaceae* or indeed similar proteins from other commercially important plant families, ie Ms41-A like proteins.

In turn the equivalent genes may be identified by hybridisation studies, restriction fragment length polymorphism (RFLP), degenerate PCR and other methods known in the art. Genes or other DNA sequences, whether natural, engineered or synthetic, encoding closely equivalent proteins may for example hybridise under stringent conditions (such as at approximately 35°C to 65°C in a salt solution of approximately 0.9 molar) to the *Arabidopsis* gene, or fragments of it of, for example, 10, 20, 50 or 100 nucleotides. A 15-20 nucleotide probe would be appropriate under many circumstances.

In the context of the present invention, "Nucleic acid which encodes" includes all nucleic acid, eg DNA sequences which will, when expressed, give rise to the protein. Examples of such DNA sequences include, but are not limited to, ones which comprise non-coding regions, e.g introns, sequences which include leader sequences and/or signal sequences, or simply comprise a coding sequence for the protein. The skilled person will also appreciate that, due to codon degeneracy, there will, for example, be a number of DNA sequences capable of coding for the Ms41-A protein or a Ms41-A like protein.

In general, the Nucleic acid of the invention will comprise at least a direct coding sequence for the protein as well as a promoter and transcription termination sequence. The promoter can itself comprise

only those sequences, or elements, necessary for the correct initiation of transcription (which regions can be described as transcription initiation regions, for instance), or, alternatively, it can include regions of sequence which are not directly involved in the initiation of transcription, i.e. a complete promoter can be employed.

A preferred coding sequence described in this specification is from *Arabidopsis* and can be isolated by methods known in the art, for example by (a) synthesising cDNA from mRNA isolated from *Arabidopsis*, (b) isolating this cDNA. This cDNA can, in turn, be used (c) as a probe to identify regions of the plant genome of a chosen member of another plant species, eg Maize, that encode mRNA of interest and (d) identifying the upstream (5') regulatory regions that contain the promoter of this DNA.

A particularly preferred DNA sequence is that shown in figure 3, and more particularly, the sequence shown in figure 3 which commences with the base pair labelled 1, as will subsequently be described in the examples. Those skilled in the art will, with the information given in this specification, be able to identify with sufficient precision the coding regions and to isolate and/or recombine DNA containing them.

The Nucleic acid of the invention can be used to confer male sterility on plants. For instance, the recessive form of the gene, ie ms41-A can be used to transform a plant. Alternatively, the dominant form, ie Ms41-A can be downregulated in some way.

As discussed herein, the Nucleic acid can include a

promoter, and to increase the liklihood of male sterility being conferred it is possible to use promoters which drive expression in particular plant tissues which are involved in the control of fertility. Examples of such promoters are those which are tapetum-specific, for example a Brassicaceae A3 or A9 promoter, described in WO-A-9211379, and the A6 promoter described in WO-A-9302197. Both WO-A-9211379 and WO-A-9302197 are hereby incorporated by reference.

Because of the natural specificity of the regulation of expression of the Ms41-A or Ms41-A like gene, it is not necessary for the Ms41-A promoter to be linked to specific disrupter DNA to provide a useful male-sterility system (although it can be); non-specific disrupter DNA can be used.

Ms41-A like promoters from other plant species, eg from Maize, and modified Ms41-A promoters can be used, and if necessary located or identified and isolated as described above for the Ms41-A coding sequences, *mutatis mutandis*.

Ms41-A or Ms41-A like promoter-containing DNA in accordance with the invention can, as indicated above, be used to confer male sterility on plants, particularly those belonging to the family *Brassicaceae*, in a variety of ways as will be discussed below. In an important embodiment of the invention, therefore, a promoter as described above is operatively linked to DNA which, when expressed, causes male sterility.

Since an effective sterility system is complete, propagation of the seed parent must proceed either by asexual means or via the pollination of the male-sterile

by an isogenic male-fertile line, and the subsequent identification or selection of male sterile plants among the offspring. Where vegetative propagation is practical, the present invention forms a complete system for hybrid production. Where fertility restoration is necessary to produce a seed crop, the present invention forms the basis of a new male sterility system. In some seed crops where the level of cross pollination is high, seed mixtures may enable restoration to be bypassed. The male sterility will be particularly useful in crops where restoration of fertility is not required, such as in the vegetable *Brassica* spp., and such other edible plants as lettuce, spinach, and onions.

Nucleic acid in accordance with the invention and incorporating the Ms41-A or Ms41-A like promoter can drive male sterility DNA thereby producing male sterile plants, which can be used in hybrid production.

A construct comprising a promoter operatively linked to a male sterility DNA can be transformed into plants (particularly those of the genus *Brassica*, but also other genera such as *Nicotiana* and *Hordeum*) by methods which may be well known in themselves. This transformation results in the production of plants, the cells of which contain a foreign chimeric DNA sequence composed of the promoter and a male sterility DNA. Male-sterility DNA encodes an RNA, protein or polypeptide which, when produced or over-produced in a stamen cell of the plant, prevents the normal development of the stamen cell.
The Ms41-A or Ms41-A like promoter may be used to drive a variety of male sterility DNA sequences which code for RNAs, proteins or polypeptides which bring about the failure of mechanisms to produce viable male gametes. The

invention is not limited by the sequence driven, but a number of classes and particular examples of male sterility promoter-drivable sequences are preferred.

For example, the drivable male sterility DNA may encode a lytic enzyme. The lytic enzyme may cause degradation of one or more biologically important molecules, such as macromolecules including nucleic acid, protein (or glycoprotein), carbohydrate and (in some circumstances) lipid.

Ribonuclease (such as RNase T1 and barnase) are examples of enzymes which cause lysis of RNA. Examples of enzymes which lyse DNA include exonucleases and endonucleases, whether site-specific such as EcoRI or non-site-specific.

Actinidin is an example of a protease, DNA coding for which can be suitable male sterility DNA. Other examples include papain zymogen and papain active protein.

Lipases whose corresponding nucleic acids may be useful as male sterility DNAs include phospholipase A_2.

Male sterility DNA does not have to encode a lytic enzyme. Other examples of male sterility DNA encode enzymes which catalyse the synthesis of phytohormones, such as isopentyl transferase, which is involved in cytokinin synthesis, and one or more of the enzymes involved in the synthesis of auxin. DNA coding for a lipoxygenase or other enzymes having a deleterious effect may also be used.

As mentioned above, one way to confer male sterility will be to downregulate the Ms41-A or Ms41-A like gene. This

could be achieved by the use of antisense DNA. Introducing the coding region of a gene in the reverse orientation to that found in nature can result in the down-regulation of the gene and hence the production of less or none of the gene product. The RNA transcribed from antisense DNA is capable of binding to, and destroying the function of, a sense RNA version of the sequence normally found in the cell thereby disrupting function.

It is not crucial for antisense DNA solely to be transcribed at the time when the natural sense transcription product is being produced. Antisense RNA will in general only bind with its sense complementary strand, and so will only have its toxic effect when the sense RNA is transcribed. Antisense DNA corresponding to some or all of the DNA encoding the Ms41-A or Ms41-A like gene product may therefore be produced not only while the gene is being expressed. Such antisense DNA may be expressed constitutively, under the control of any appropriate promoter.

It is also the case that one may wish to restore male fertility in later generations. this can also be achieved using antisense nucleic acid, eg nucleic acid which is antisense for a DNA molecule encoding ms41-A.

Thus, in a second aspect, the present invention provides Antisense nucleic acid which includes a transcribable strand of DNA complementary to at least a part of a DNA molecule of the invention.

In one embodiment of this aspect the antisense nucleic acid is under the control of a constitutive promoter,

such as the CaMV35S promoter.

A still further example of male sterility DNA encodes an RNA enzyme (known as a ribozyme) capable of highly specific cleavage against a given target sequence (Haseloff and Gerlach *Nature* **334** 585-591 (1988)). Like antisense DNA, ribozyme DNA (coding in this instance for a ribozyme which is targeted against the RNA encoded by the Ms41-A or Ms41-A like gene) does not have to be expressed only at the time of expression of the Ms41-A or Ms41-A like gene. Again, it may be possible to use any appropriate promoter to drive ribozyme-encoding DNA, including one which is adapted for constitutive expression.

According to a further aspect of the invention, there is therefore provided DNA encoding a ribozyme capable of specific cleavage of RNA encoded by a DNA molecule of the invention. Such ribozyme-encoding DNA would be useful in conferring male sterility on members of, eg the family *Brassicaceae*.

In addition, there are other useful methods which can be employed for the downregulation of the Ms41-A or Ms41-A like DNA sequences. Some examples of these are as follows:

i) expression of an antibody or antibodies, domains or fragments thereof against the Ms41-A or a Ms41-A like protein;

ii) expression of mutant versions of the Ms41-A or of a Ms41-A like protein which may interfere with the function of the normal protein;

iii) by creation of mutations in the Ms41-A sequence or the the Ms41-a like sequence with the result that mutant plants can be used in the recessive AMS system as hereinbefore described; and

iv) expression of mRNA binding proteins that will interfere specifically with Ms41-A or Ms41-A like transcription.

In preferred embodiments of DNA sequences of this invention 3' transcription regulation signals, including a polyadenylation signal, may be provided. Preferred 3' transcription regulation signals are derived from the Cauliflower Mosaic Virus 35S gene. It should be recognised that other 3' transcription regulation signals could also be used.

Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will be present; however, DNA in accordance with the invention will generally be expressed in plant cells, and so microbial host expression would not be among the primary objectives of the invention, although it is not ruled out. Vectors not including regulatory sequences are useful as cloning vectors.

Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation. According to another aspect of the invention, there is therefore provided a host cell transfected or transformed with DNA as described above.

DNA in accordance with the invention can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but recombinant DNA technology forms the method of choice.

Ultimately, DNA in accordance with the invention (whether (i) Ms41-A gene, ms41-A gene, Ms41-A like gene or ms41-A like gene (ii) antisense DNA to any option listed in i), ribozyme DNA targeted to RNA for any option listed in i) or DNA comprising a promoter as described herein used to drive expression of a disrupter sequence, eg encoding Barnase) will be introduced into plant cells, by any suitable means.

According to a further aspect of the invention, there is provided a plant cell including DNA in accordance with the invention as described above.

Preferably, DNA is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign DNA could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example where the recipient plant is monocotyledenous. Any other method that provides for the stable incorporation of the

DNA within the nuclear DNA of any plant cell of any species would also be suitable. This includes species of plant which are not currently capable of genetic transformation.

Preferably DNA in accordance with the invention also contains a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign DNA to be easily distinguished from other plants that do not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al*, *EMBO J.* 2, 987-995 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the tapetum, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. However any other suitable second promoter could be used.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods. When the transformed plant flowers it can be seen to be male sterile by the inability to produce viable pollen. Where pollen is produced it can be confirmed to be non-viable by the inability to effect seed set on a recipient plant.

Preferred features of each aspect of the invention are as

for each other aspect *mutatis mutandis*.

The invention will now be illustrated by a number of non-limiting examples, which refer to the accompanying drawings, in which:

FIGURE 1: shows a Southern Blot of *Hind*III-cut genomic DNA from 21 *ms*41-A plants demonstrating linkage of the 35S-Ac element to *ms*41-A;

FIGURE 2: shows a schematic diagram of the region containing the *MS*41-A locus cloned in lambda MSE3. The position of insertion of the 35S-Ac is indicated; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sae*I;

FIGURE 3: shows the genomic DNA sequence of the *MS*41-A gene. The sequence is numbered from the putative transcriptional start point of the *MS*41-A message. The predicted amino-acid sequence of *MS*41-A is shown together with the restriction sites;

FIGURE 4: shows the predicted amino acid sequence of *MS*41-A;

FIGURE 5: shows the oligonucleotides used to examine excision events of 35S-Ac from the *ms*41-A locus;

FIGURE 6: shows DNA sequences left by 35S-Ac excision events at the *ms*41-A locus;

FIGURE 7: shows a diagram of the *MS*41-A

promoter-GUS and *MS*41-A promoter-Barnase chimeric genes;

FIGURE 8: shows a diagram of the *MS*41-A promoter-antisense *MS*41-A and CaMV 35S promoter-antisense and sense *MS*41-A chimeric genes;

FIGURE 9: shows sequence alignments of proteins related to *MS*41-A;

FIGURE 10: shows a partial DNA sequence and predicted amino acid translation of *Zm*41-A;

FIGURE 11: shows a dendrogram of *MS*41-A related sequences;

FIGURE 12: shows the nucleotide sequence of the Z31 *Zm*41-A gene. The portion of the sequence corresponding to putative coding region is shown in bold type capital lettters. ◆ indicates putative first methionine deduced in frame with cDNA *Zm*41-A and 5'RACE products. * indicates the start of the longest 5'RACE product. ▼ indicates the start of *Zm*41-A cDNA. 12 exons are present and the translation is stopped in exon 11, the stop codon is TGA (□). Non spliced DNA present in some RACE products is underlined;

FIGURE 13: shows restriction maps of Z31, Z33 and Z35 genomic clones isolated with cDNA of *Zm*41-A. EI, HIII, NI and SI indicate restiction sites of endonucleases *Eco*RI, *Hind*III, *Nco*I and

SalI, respectively. * indicates the start of the longest RACE product. ▼ indicates the start of *Zm*41-A cDNA. Dotted lines indicate homologous regions and Δ indicates deletions;

FIGURE 14: shows clustal V alignment between the protein deduced from the *Zm*41-A cDNA and from the genomic longest open reading frame of Z31;

FIGURE 15: shows the nucleotide sequence of the Z33 *Zm*41-A gene. The portion of the sequence corresponding to DNA transcription is shown in bold type capital letters. Non spliced DNA present in some RACE products is underlined. This gene is truncated and only exons 3,5 and 6 are present; and

FIGURE 16: shows the nucleotide sequence of the Z35 *Zm*41-A gene. The portion of the sequence corresponding to DNA transcription is shown in bold type capital letters. Non spliced DNA present in some RACE products is underlined. This gene is truncated and only exons 3,4,5 and 6 are present.

Example 1

Isolation of a gene required for male fertility in *Arabidopsis thaliana*

i) Isolation and phenotype of the ms41-A male sterile mutant.

The method used to identify a gene required for male fertility in *Arabidopsis thaliana* was transposon tagging. This method is a powerful technique for isolating genes which encode unknown products, allowing genes identified only by their mutant phenotype, to be cloned. *Arabidopsis thaliana* is a widely used model species that is an ideal plant for transposon tagging of genes, since it is a transformable diploid with a very small genome. Thus the chance of tagging desired genes is maximised. Additionally *Arabidopsis* is a *Brassicaceae* and is thus very closely related to important crop plants such as *Brassica napus* (Oil Seed Rape).

Transposon tagging was achieved by transformation of C24 *Arabidopsis* roots with modified autonomous Ac elements from Maize: D Ac and 35S Ac inserted into the leader of the GUS reporter gene in the reverse orientation (Constructs described in Finnegan *et al.*, *Plant Molecular Biology*, **22**:625-633 (1993)). (As this work was in progress the first reports of gene tagging with similar Ac elements in heterologous plant species were published; a pH controlling gene from Petunia: Chuck *et al.*, *Plant Cell*, **5**:371-378 (1993)); the *Arabidopsis* DRL1 locus: Bancroft *et al.*, *Plant Cell*, **5**:631-638 (1993)) and the *Arabidopsis* Albino gene (Long *et al.*, *Proceedings of the National Academy of Sciences U.S.A.*, **90**:10370-10374 (1993)).

Transformed plants were regenerated and the T2 progeny analysed for GUS activity and by molecular analysis. This demonstrated that the 35S Ac transposed quite efficiently (in 30% to 40% of progeny). The T3 progeny families derived from 279 selected T1 plants were then visually screened for mutants affected in male sterility.

A few fertility-reduced or sterile plants were recovered, some possessing additional abnormalities. A male sterile mutant (ms41-A) which appeared in family 41 had collapsed anthers with empty locules. Only one sterile plant was recovered from more than 2000 T3 siblings in this family. After cross-pollination with wild type pollen, elongation of siliques was observed, confirming that female fertility is unaffected by the mutation.

From the above cross 21 F1 individuals were grown and allowed to self pollinate to produce F2 seed ; all the F1 plants were completely fertile suggesting that the mutation is recessive. The first analysis of 6 different F2 populations confirmed the recessive character of the mutation, as male sterility reappeared in a small proportion of each F2 population, with all other siblings presenting a wild type phenotype. Moreover, the vegetative development of the male sterile plants was identical to wild type C24 *Arabidopsis*. The observed frequency of male transmission of the mutation suggests a non-classical mendelian inheritance for a single recessive mutation - the frequencies of mutant plants in the F2 populations were: 16.8 ; 13.0 ; 11.9 ; 12.7 ; 15.4 and 17.0 %. The expected frequency of mutant plants is 25 % or a 3 to 1 ratio of wild type to mutant plants. In this case there is a ratio of approximately 7 to 1 wild type to mutant plants. A homogeneity test on the data of the 6 F2 populations presented concludes that there is homogenous transmission of the male sterile phenotype (Chi square with 5 degrees of freedom = 8.69, $0.10<P<0.20$).

Proof of reduced transmission of Ms41-A through the male

gametophyte was obtained by genetic mapping of Ms41-A. The hypothesis was that markers genetically linked to Ms41-A but present on the homologous chromosome (in repulsion) on a F1 cross with an Ms41-A plant should be over-represented in the derived F2 population. The F1 crosses were made with 5 tester lines, one for each chromosome, constructed by Marteen Korneef (described in; O'Brian S.T. (ed) Genetic maps of complex genomes, Book 6, Plant Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 94-97 (1990)), and linkage of Ms41-A was demonstrated with markers on the lower part of chromosome 1. Compiled recombination data of 2 populations (476 and 540 individuals) were analysed by the Map Maker software version 2 (Lander *et al.*, *Genetics*, **121**:174-181 (1987))).

Ms41-A is between apetala 1 (8.1 cM) and glabra 2 (9.8 cM) and 40.2 cM away from than chlorina 1. In the first F2 population, the deficit of Ms41-A plants was observed as before (14.7% of plants were male sterile) and it was correlated with the expected increase of apetala 1 and glabra 2 plants (29 % and 31.5 % respectively) ; the most distal marker, chlorina 1 behaves quite normally (22.3 %). In the second F2, where the penetrance of the Ms41-A is less affected (18.3 %), the over representation is not as prevalent (as expected); only the proportion of glabra 2 plants appears to be slightly increased (27.2 %).

Microscopic observations of microsporogenesis in the male sterile Ms41-A plants revealed that the tetrads release abnormal microspores which degenerate rapidly. By aniline blue staining the tetrads appear abnormal with irregular shaped cells and with great variation in cell size. Moreover there is a mixed population of meiocytes, dyads

(a stage not usually observed in *Arabidopsis*) and tetrads in the same anther. The defect apparently lies just before or during meiosis.Cytological observations on fixed young anther buds reinforce this finding, since at meiosis the meiocytes are affected but the tapetum behaves normally. No differences were observed cytologically between the Ms41-A heterozygote and wild type plants.

One other gene required for male-fertility (also in *Arabidopsis*) has been described previously (Aarts *et al.*, *Nature*, **363**:715-717 (1993)). Plants with a mutation in this gene (Ms2) were grown together with Ms41-A plants. In certain conditions , especially after the plants had been flowering for a long time the ms2 but not the Ms41-A plants reverted to male fertility.

ii) Linkage of a transposed 35S Ac with the mutant phenotype

To determine if the Ms41-A mutation was due to the insertion of a 35S-Ac element, HindIII-cut DNA from five Ms41-A F1 individuals was analysed by Southern blotting using a 5'Ac fragment (2.5 Kb EcoR I fragment from pBGS335RI (Finnegan *et al.*, *Plant Molecular Biology*, **22**: 625-633 (1993)) as a probe. Two identical Ac bands were present in the five mutant plants :

- the internal Ac Hind III 1.6 kb band and
- a junction 3' Ac band of approximately 2.8 kb, which differs from the expected non-transposed 35S Ac (2.1 kb).

This indicates the presence of only one 35S Ac element which has transposed in the parental male sterile plant,

or more likely in its parents. To determine linkage between this 35S Ac element and the Ms41-A phenotype, 24 Ms41-A plants from each of 6 different F2 populations were analysed by PCR for the presence of the Ac element using oligonucleotides:-

5' H (5' AAGGATCCTGGCAAAGACATAAATC 3') and

Ac12 (5' AGATGCTGCTACCCAATCTTTTGTGC 3').

The results were as follows :

F2 41-A-A	23	positives out of 24
F2 41-A-B	5	"
F2 41-A-C	23	"
F2 41-A-D	10	"
F2 41-A-E	24	"
F2 41-A-F	3	"

If the Ac element is linked to Ms41-A all male sterile plants should have the Ac element, however if the Ac is not linked only 3/4 of Ms41-A plants should have the Ac element. The results obtained indicate complete linkage only in the 41-A-E population. The lack of linkage in the other populations may be due to frequent imprecise excision of the Ac element from the Ms41-A locus leaving a mutation in Ms41-A.

To confirm linkage, the most stable population, 41-A-E, was analysed by Southern blotting with a probe that contained both a region of the transposed Ac element and 3' flanking plant DNA. To generate this probe DNA from a Ms41-A plant was digested with SspI, religated and amplified by PCR using Ac oligonucleotides:-

Ac 11 (5' CGTATCGGTTTTCGATTACCGTATT 3') and

Ac 12 (5' AGATGCTGCTACCCAATCTTTTGTGC 3').

The 1.1kb inverse PCR (IPCR) fragment generated contained 500 bp of Ac and the remainder consisted of 3' flanking

Arabidopsis DNA.

DNA from plants of the F2 population 41-A-E was digested with HindIII and probed with the 3' IPCR fragment. 21 new F2 mutant individuals and 28 male fertile F2 plants were analysed, the selfed progenies of the latter were checked for the presence of mutant plants revealing that 15 of the 28 were heterozygous for Ms41-A. All of the 21 mutant plants (Figure 1) and those heterozygotes segregating the mutation in the F3 showed the same transposed 35S Ac revealed by the 2.8 kb specific band and the Ac internal 1.6 kb band. A 3.3 kb band, corresponding to the wild type allele is detectable in most of the F2 mutants; this is probably due to to somatic excision of Ac and confirms that the transposed Ac element is still active. These results confirm that the 35S Ac is located in or in the vicinity of the Ms41-A gene.

iii) Genomic clones and cDNAs of the Ms41-A gene

Two different genomic libraries - one MboI partial library in EMBL 3A (Clontech) and one HindIII partial in Lambda Dash II (T. Pelissier, S.Tutois and G. Picard, unpublished) were screened with the 3' IPCR cloned product. Four different clones spanning the mutated region, were characterised by Southern analysis. One of them, lambda MSE3, which spans the transposon insertion site, was used for fine mapping. It contains the IPCR hybridising fragments detected on a genomic Southern (HindIII 3.3 kb, SspI 1.8 kb and PstI 4 kb). The entire plant DNA insert in MSE3 is contained on 4 SalI fragments; S1 (5kb), S2 (4.9kb), S3 (4.3kb) and S4 (2.3kb) (Figure 2). The S3 fragment contains the plant DNA from the IPCR product.

After sequencing the IPCR product to determine the plant sequence 3' of the Ac element, more than 5000 bp of genomic sequence was obtained from MSE3 (3100 bp from the 5' Ac flanking region and 1900 bp at the 3'). The genomic sequence is presented in figure 3 and is indexed according to the putative transcription initiation site determined by 5' RACE (see below). One of the SalI sites of the fragment S3 is positioned at 2061 bp the other one is situated 5' upstream an EcoRI site (-1753 bp) and has not been sequenced. The transposon is inserted at position +318 bp.

To identify mRNAs expressed in the region of the transposon insertion site, three *Arabidopsis* cDNA libraries were probed with either the S1 or S3 fragments; a developing flower buds library (young buds) (Weigel *et al.*, *Cell*, **69**:843-859 (1992)), a library from flowers at late stages (after stage 10) (Hofte *et al.*, *Plant Journal*, **4**:1051-1061 (1993)) and an immature siliques library (Giraudat *et al.*, *Plant Cell*, **4**:1251-1261 (1992)).

Two classes of cDNAs were recovered with the S3 fragment as a probe and characterised.

- a 1.9 kb cDNA (W11), isolated from the developing flower buds library. Its 3' end is located 1.5 kb upstream of the 3' 35S Ac end, suggesting that it is not linked to the Ms41-A phenotype. Sequencing of the extremities revealed that the EcoRI site (-1753bp in figure 3) is present in the 3' part of this mRNA.

- a 0.8 kb cDNA (G6), isolated from the immature siliques library but also present in the developing flower buds library. Comparison of G6 and genomic sequences shows

that the transposon insertion site is 1440 bp upstream of the 5' end of the longest G6 cDNA (861 bp). In addition, the lack of a methionine codon in the 5' sequence of G6 indicated that this cDNA was not full-length. Further attempts at obtaining longer cDNAs from the three libraries were unsuccessful.

Another cDNA (A6) of approximately 1Kb was isolated using the S1 fragment as a probe. It maps downstream of the G6 message.

Out of the 3 transcription units in the vicinity of the transposon insertion site, the best candidate for the Ms41-A mRNA was that corresponding to G6. To obtain a full-length G6 cDNA, primers were designed to the 5' end of the longest G6 cDNA and used in a 5' RACE reaction (5' AmpliFinder kit, Clontech). This proved unsuccessful, probably due to the 5' end of G6 lying far upstream of the longest cDNA obtained. Therefore primers were designed to regions of the genomic sequence that were upstream of the 5' end of the longest G6 cDNA. These, in combination with primers designed to the G6 cDNA, were used in RT-PCR reactions to define the extent of the G6 transcribed region. Results obtained suggested that the G6 message was at least 1 kb longer than the longest G6 cDNA obtained, and that the upstream sequence contained an intron of about 450 bp.

The G6 transcriptional start site was finally mapped by 5' RACE using primers Z3
(5' TTATCATCAACATCGCCATCGAATCTGCCG 3', positions 494-464 bp in Figure 3);
and W1 (5' AAAGTAGTAAACCCTAGAG 3', positions 279-260 bp). RT-PCR was then used to recover a nearly full-length G6

message. Comparison of the G6 and genomic sequences shows that the first ATG is situated at position 157 bp; thus G6 putatively encodes a protein of 584 amino acids (Figure 4). Over the region of overlap the cDNA and genomic DNA sequences were identical. This deduced protein has no significant homology to proteins of known function on the Genebank, EMBL and NBRF databases. The coding sequence consists of three exons, the first of which has been disrupted by the insertion of the 35 Ac element at amino acid position 54 in the Ms41-A mutant. This is strong evidence that G6 corresponds to Ms41-A. Final confirmation was obtained by analysis of phenotypes and DNA sequences around the Ac insertion site in Ms41-A progeny plants in which the 35S Ac element has excised.

To induce somatic exision of the 35S Ac element, plants were regenerated from liquid root cultures from single individuals derived from two different test-crosses. These crosses where between plants (A and B) that had only one Ac element but were still male sterile due to imprecise exision of the other Ac element, and male fertile plants that were heterozygous for Ms41-A: 35S Ac. This material was chosen because of the higher percentage of male sterile plants (40% instead of 20%, 50% instead of 25%?) than in a normal F2 population. Regenerants from clones representing male sterile plants were scored for male fertility. Numerous completely fertile plants were obtained from some individuals, however from 5 different regenerated plants from 4 different individuals, 7 different "revertant siliques" were obtained.

DNA from revertant plants or from progeny from "revertant siliques" was analysed by PCR for excision of the Ac element and PCR products cloned to determine the sequence

left by the Ac element (footprint). The oligonucleotides presented in Figure 5 were used : Ac 11 with W2 for the presence of the 3' junction, Ac 14 with G6 5'-11 for the 5' junction and W2 with G6 5'-11 or with Z3 for the excision allele(s). The PCR fragments derived from W2 with G6 5'-11 or with Z3 were cloned in the pGEM-T vector (Promega) and sequenced for all revertants. Previously junction products were sequenced confirming the presence of the typical target duplicated sequence of 8 base pairs : CTCCTCTC (positions 311 to 318 in Figure 3).

The genotypes of 7 revertant plants or sectors were determined and are presented in figure 5. For all of them an allele restoring the open reading frame is observed which is the same as the wild type in 4 cases , a 3 bp insertion in 2 cases and a 6 bp insertion in one case. Footprints destroying the coding phase are observed in different revertants and also in the female parents (2 different 7 bp insertions and 2 different 5 bp insertion, and one with the addition of a 9 bp insertion which also introduces an in frame, TGA, stop codon). Their presence is always associated with segregation of male sterile individuals in the progeny. These results demonstrate that the Ms41-A protein has a determinant role in male fertility and that the Ms41-A gene has been tagged with the 35S Ac element.

iv) Ms41-A genetic mapping

Classical genetic mapping of Ms41-A with visual phenotypic markers has been described previously in section i) of this example. It places the Ms41-A locus near the bottom of chromosome 1. To determine if the Ms41-A mutation has been isolated previously in

Arabidopsis the mutation was mapped more precisely using recombinant inbred lines made by Caroline Dean (Lister *et al.*, *Plant Journal*, 4:745-750 (1993)). This method requires the identification of restriction enzyme fragment length polymorphisms (RFLPs) between the two parental lines (Columbia and Landsburg erecta) which are in, or near the Ms41-A locus. Polymorphisms were not found in Ms41-A or 5' of it, however the downstream cDNA, 6A, gives a HhaI polymorphism. Results, processed by MapMaker version 2, have positioned Ms41-A near the marker m532 (1.3 cM) and marker g17311 (4.6 cM). Those RFLP markers are situated on chromosome 1 close to the ADH locus, and map in the vicinity of glabrous 2 and apetala 1 on the integrated Arabidopsis genetic map (Hauge *et al.*, *Plant Journal*, 3:745-754 (1993)).

Ms41-A is a new male-sterile mutant. It is not allelic to ms1 (Van der Veen and Wirtz, *Euphytica*, 17: 371-XXX (1968)) ms3, ms5, ms10, ms11 or ms12 (Chaudhury 1993). It is also different to the Ms2 gene (Aarts *et al.*, *supra*).

v) Abundance of the Ms41-A message

Ms41-A is expressed in 7 day old seedlings, in young floral buds and in immature siliques (cDNA libraries and RT-PCR data). The mRNA could not be detected in these tissues by Northern blotting using poly A+ mRNA which had been used successfully in RT-PCR analysis for the Ms41-A message. Thus the Ms41-A message appears to be of very low abundance; approximately 10 fold lower than another message required for male ferility in *Arabidopsis*, Ms2, in the same cDNA library (1 out of 12 000 plaques for Ms2 (Aarts *et al.*, *supra*) versus 1 out of 125 000 for

Ms41-A).

Example 2

Isolation of the Ms41-A promoter and fusion to the ß-Glucuronidase (GUS) reporter gene

To attempt to determine the extent of utility of the Ms41-A promoter in male sterilty systems putative Ms41-A promoter fragments were linked to the reporter gene GUS and transformed into *Arabidopsis* and tobacco. This will reveal more precisely the spatial and temporal expression patterns of the Ms41-A gene and determine whether the low abundance of the Ms41-A transcript is due to weak expression or transcript instability.

Two promoter fragments, -903 (Hind III) to +79 (Short promoter) and -1753 (EcoR I) to +79 (Long promoter), have been fused to the GUS gene (transcriptional fusions) to produce the binary vectors pBIOS 176 and pBIOS 177 (Figure 7).

These plasmids were constructed as follows:-
The primers Y7 (positions -1799 to -1782 in Figure 3)
5' CCTAACTTTCTTTGCGGC 3'
and W3 Xba (positions 84 to 59 in Figure 3)
5' GATCTAGACCGTGATGTCTTAGAAGG 3'
were used in a PCR to recover a 1883 bp Ms41-A promoter fragment. This was cloned into the vector pGEM-T (Promega) forming pS11. This plasmid was introduced into a dam, dcm minus *E.coli* strain (SCS 110) thus allowing the XbaI restriction enzyme to cleave the XbaI site. The 985 bp HindIII, XbaI fragment of pS11 was cloned between the HindIII and XbaI sites of pBI121 (replacing the 35S

CaMV promoter of this plasmid) forming plasmid pBIOS176. The 1853 bp EcoRI, XbaI fragment of p511 was cloned between the EcoRI and XbaI sites of pBIOS4 (a derivative of pBI121), replacing the 35S CaMV promoter of this plasmid, forming plasmid pBIOS177.

To construct pBIOS4, pBI121 was digested with EcoRI, the ends filled using Klenow polymerase and then religated forming pBIOS5. This plasmid was digested with HindIII, the ends filled using Klenow and an EcoRI linker ligated into the destroyed HindIII site, forming pBIOS4.

pBIOS176 and pBIOS177 were transformed into *Arabidopsis* and tobacco. The larger promoter fragment is predicted to contain the entire Ms41-A promoter region since the EcoRI site lies with the 3' end of the W11 transcript.

Arabidopsis results:-

a) Short promoter:- Histochemical staining reveals that GUS activity is observed in most tissues and is especially high in callus, (strong blue staining is detectable after a few hours in X-GLUC (5-bromo-4-chloro-3-indolyl glucuronide).

b) Long promoter:- GUS activity was seen in callus, but no obvious blue staining was observed in the vegetative parts of primary transformants. However 75% of the 40 transformants had significant GUS activity in anthers. In the floral buds observed, GUS expression is detected just after the breakdown of the callose wall (floral stage 10); expression appears to be located initially in the tapetum and subsequently in the microspores. GUS activity is still present in mature pollen. However it is possible

that there is also GUS activity in the microsporocytes and tetrad microspores since the GUS substrate may not pentrate the thick callose walls surrounding the microsporocytes and tetrads.

Similar staining experiments were done with plants containing the 3 tapetum-specific promoter fusions - TA29 (Koltunow *et al.*, *Plant Cell*, **2**:1201-1224 (1990)), A6 (Hird *et al.*, *Plant Journal*, **4**:1023-1033 (1993)) and A9 (Paul *et al.*, *Plant Molecular Biology*, **19**:611-622 (1992)) and with the microspore/pollen promoter LAT 52 (Twell *et al.*, *Molecular and General Genetics*, **217**:240-245 (1989)).

A9 is definitely the earliest and with the A6 promoter, GUS is expressed when tetrads are visible; by contrast the TA 29 promoter gives expression at roughly at the same time as Ms41-A; the latter also shows earlier expression in microspores than LAT 52. In seedlings of 5 out of 7 transformed plants, very low levels of GUS expression is detected in aerial parts.

Tobacco results:-

a) Short promoter:- GUS expression appears to be constitutive.

b) Long promoter:- Results were similar to those observed in *Arabidopsis*, ie expression is largly confined to the tapetum, microspores and pollen of the anther. Very low GUS expression was seen in the aerial parts of seedlings, however no expression was detected in callus.

It appears that expression from the long promoter matches that of the Ms41-A gene, with very low level

"consitutive" expression. Expression in the anther is much stronger than predicted by the abundance of Ms41-A transcript in floral parts indicating that the Ms41-A message may be very unstable. Higher level constitutive expression observed from the short promoter suggests that there a constitutive silencer is present in the upstream region of the promoter between posititions -1635 to -900 bp. The conserved pattern of expression of the long promoter between tobacco and *Arabidopsis* suggests that the long promoter will be useful in male sterility systems in a wide range of plant species. Examples 3 and 4 below demonstrate the use of the long Ms41-A promoter in male sterility systems.

Example 3

Expression of Barnase from the Ms41-A promoter in Tobacco and Maize

The timing of expression of the Ms41-A promoter in the tapetum is similar to that seen from the tobacco TA29 promoter, thus fusion to cytotoxins such as Diptheria toxin A (Thorsness *et al.*, *Developmental Biology*, **143**: 173-184 (1991)) and Barnase (Mariani *et al.*, *Nature*, **347**: 737-741 (1990)) will ablate the anther tapetum leading to complete male sterility. Thus the long Ms41-A promoter is linked to Barnase. A 1kb XbaI, HindIII (filled) fragment encoding Barnase is excised from pWP127 (Paul *et al.*, *supra*) and cloned between the XbaI and SstI (filled) sites of pBIOS177 forming pBIOS 177-Barnase (Figure 7).

This plasmid is used to regenerate tobacco and Maize transformants that are male sterile. Although the weak "consitutive" expression of the Ms41-A promoter should

prevent recovery of such plants, it is likely that these plants have reduced Ms41-A promoter expression. Thus no significant expression of Barnase occurs in vegetative tissues whereas expression is sufficient to cause tapetal cell death and male sterility.

Example 4

Expression of antisense Ms41-A from the Ms41-A promoter in *Arabidopsis*

The Ms41-A promoter can be used to downregulate the expression of genes essential for tapetal function thus causing complete male sterility. Downregulation can be achieved by expression from the Ms41-A promoter of antisense or sense fragments of the target gene or by expression of ribozymes which will cleave the target gene transcript. Such a target gene is Ms41-A. To construct an Ms41-A promoter- Ms41-A antisense chimeric gene, RT-PCR is used to generate a 1923 bp Ms41-A fragment from young *Arabidopsis* floral buds mRNA. The primers used are:-
W3 Bam, 5' CGGATCCTTCTAAGACATCACG 3' (positions 54-75, Figure 3) and
3'2, 5' AATGTACTACTACTACTACTTAGGAC 3' (positions 3001-2976, Figure 3).

This PCR fragment is cloned into pGEM-T forming p542, such that the 5' end of MS41-A is adjacent to the ApaI site of pGEM-T (Figure 7). The MS41-A SpeI, ApaI (filled using T4 DNA polymerase) fragment is cloned between the XbaI and SstI (filled) sites of pBIOS177, thus replacing the GUS gene of pBIOS177 and forming pBIOS182 (Figure 8). This plasmid is used to transform *Arabidopsis*. A proportion of transformants are male sterile with a

phenotype that resembled that of the original Ms41-A mutant. Examples 5 and 7 below describe the use of the Ms41-A transcribed region in male sterility systems.

Example 5

Expression of a 35S CAMV promoter- Ms41-A antisense chimeric gene and a 35S CaMV promoter Ms41-A sense chimeric gene in *Arabidopsis*

As described in Example 4, downregulation of the Ms41-A gene by expression of Ms41-A antisense fragments, sense fragments or ribozymes, each driven from the Ms41-A promoter will lead to male sterility. However any promoter that has the appropriate pattern of expression, ie is active in microsporocyte and/or tapetal cells of the anther at the time of Ms41-A expression, may be used to downregulate Ms41-A and cause male sterility. Thus a CaMV 35S promoter is linked to an antisense Ms41-A fragment and to a sense Ms41-A fragment. The antisense construct is obtained by cloning the ApaI (filled), SpeI p542 MS41-A fragment between the XbaI and SstI (filled) sites of pBIOS4 forming pBIOS188 (Figure 8).

The sense construct is obtained by cloning the ApaI (filled), SstI p542 MS41-A fragment between the SmaI and SstI sites of pBIOS4 forming pBIOS186 (Figure 8). These plasmids are transformed into *Arabidopisis*. A proportion of the antisense and sense transformants are male sterile with a phenotype similar to that of the original Ms41-A mutant plant.

Example 6

Isolation of a Ms41-A orthologue from Maize

Most methods to use the coding region of the Ms41-A in a male sterilty system require the isolation of the orthologous sequence either from the crop species of interest or from a close evolutionary relative. Such methods include antisense and sense supression and the use of ribozymes. The degree of evolutionary conservation between orthologous protein sequences is variable and is probably dependant on constraints on protein function. Athough orthologous protein sequences may be highly conserved, codon usage may be quite different, producing orthologous mRNA sequences that may have low homology. Thus, in order to downregulate the Maize version of Ms41-A, it is probably necessary to isolate the Maize version of Ms41-A. Given the *Arabidiopsis* Ms41-A mRNA sequence, several approaches are possible for the isolation of the Maize orthologue. Some of which are outlined below:-

The Ms41-A cDNA can be used as a probe on a Maize Northern or Southern at low stringency to see if a mRNA or genomic band hybridises. This was unsucessful indicating that these sequences are widely diverged. The *Arabidopsis* sequence can be used as a probe in more closely related species and the orthologues in turn used as further probes until the version in Maize is identified. The cloning and sequencing of such orthologues may also result in the identification of conserved areas that can be used in a degenerate PCR approach.

Antibodies to Ms41-A may also be useful since protein sequences and epitopes are generally more conserved than RNA/DNA sequences.

The approach used was to screen the Genebank and EST (Expressed Sequence Tag) databases for sequences that showed homology to the Arabidopsis Ms41-A DNA sequence. Four groups of sequences were identified according to the degree of sequence similarity. Alignments of these sequences are presented in Figure 9.

Group 1
This group contains the *Arabidopsis* Ms41-A cDNA and an EST sequence from rice OSS2204 (D40316) which was cloned from a shoot cDNA library (prepared from etiolated 8 day old seedlings).

Group 2
In this group are two pairs of almost identical *Arabidopsis* EST sequences (ATTS3975 (Z37232) and T43470) and (T21748 and R30405) which are presumably derived from the same transcripts and can be considered as two sequences. The R30405, T21748 and T43470 cDNAs were isolated from a library prepared using a mixture of RNA from various tissues. The ATTS3975 cDNA is from a library prepared from cell suspension culture. In addition, in this group is a rice cDNA isolated from a root cDNA library (seedling stage) OSR1187 (D24087).

Group 3
In this group are 3 EST sequences and 1 cDNA sequence ATTS1074 (isolated from a cycling cells cDNA library). A partial EST sequence for ATTS 1074 is on the database (Z25611) and after identification of this sequence as

similar to Ms-41A the cDNA clone was obtained and the sequence completed. The other 3 sequences are all identical or almost identical to the ATTS1074 sequence.

The cDNA clones R65265 and T44526 were isolated from a mixed RNA library. ATTS2424 is a 3' sequence EST sequence from the same cDNA clone as ATTS1074, this clone (TAI231) was isolated from a cDNA library prepared from a cell suspension culture containing cycling cells.

Group 4

This group contains sequences of 4 closely related plant transcription factors; Viviparous-1 from maize (McCarty *et al.*, *Cell*, **66**:895-905 (1991)) and rice (Hattori *et al.*, *Plant Molecular Biology*, **24**:805-810 (1994)), ABI 3 from *Arabidopsis* (Giraudat *et al.*, *Plant Cell*, **4**: 1251-1261 (1992)) and a *Phaseolus vulgaris* embryo-specific acidic transcriptional activator PvAlf (Bobb *et al.*, *Plant Journal* In press (1995)).

There is some amino-acid similarity between a region in the N-terminal of the Ms41-A protein and the proposed DNA binding domain of maize Viviparous-1. This region is highly conserved between the 4 transcription factors (>80 % amino-acid identity between all 4 sequences). This suggests that the Ms-41A protein may have DNA binding activity, although the MS41-A protein might be sorted via the ER, perhaps to be secreted, since Ms41-A has a putative signal peptide and 6 putative N glycosylation sites.

The most closely related sequence to Ms41-A identified by this analysis is the rice OSS2204 sequence. This was obtained from the rice sequencing project and used to

probe a Maize cDNA library made in Lambda UniZap (Stratagene) from polyA+ RNA isolated from pre-meiotic to meiotic-stage male inflorescences. The cDNA isolated, Zm41-A, is approximately 2.2 kb in length and has a poly A tail at it's 3' end. Approximately 300 bp of 5' prime sequence is shown in Figure 10.

This sequence shows strong similarity to the rice OSS2204 cDNA sequence (84 % identity) but is only 53% identical to the *Arabidopsis* sequence. The ORF indicated underneath the DNA sequence is similar to both the proposed OSS2204 ORF (89 % identical, 94 % similar) and the *Arabidopsis* Ms41-A protein sequence (54 % identical, 65 % similar).

A dendrogram of the Ms41-A related sequences indicates that the Zm41-A sequence falls into group 1 (Figure 11). This indicates that this cDNA is a good candidate for the maize orthologue of the *Arabidopsis* MS41A gene.

Example 7

Expression of an actin promoter- Zm41-A antisense chimeric gene in Maize

The Zm41-A cDNA is linked in an antisense orientation to a rice actin promoter. The entire Zm41-A cDNA is excised from pBluescript SK- (Stratagene) as an XhoI (filled), PstI fragment and cloned into PstI, SmaI - cut pCOR113 (McElroy *et al.*, *Molecular and General Genetics*, **231**: 150-160). This plasmid is used to transform Maize by a particle bombardment technique. A proportion of the transformants are male-sterile with a phenotype similar to that of the *Arabidopsis* Ms41-A mutant. This suggests that the Zm41-A sequence is the functional orthologue of

Ms41-A and indicates that any sequence that falls within group 1 (Figure 11) is likely to encode a functional orthologue of Ms41-A.

Example 8

Molecular characterisation of *Zm41-A* gene(s)

a) *Zm41-A* gene transcription

BY RT-PCR this transcript has been shown to be abundant in anther RNA; in leaf and tassel RNA populations it is detected at a lower level.

After comparison of the maize and *Arabidopsis* sequences it was thought that the cDNA was unlikely to be a full length clone. With the "Marathon cDNA amplification" kit (Clontech, Palo Alto, CA, USA) 5'RACE experiments were conducted on mRNA extracted from maize anthers at the meiosis stage, which yielded additional 5' sequence. Two types of 5'RACE products were obtained and sequenced, the first contained approximately 150bp of additional 5' sequence as well as a 108bp insertion at position 244 in the cDNA. The second RACE product contained approximately 130bp of additional 5' sequence. It is believed that the first RACE product may be the result of differential or incomplete splicing of the transcript resulting in a 36 amino acid insertion in the predicted peptide sequence as well as the 52 additional amino acids at the N terminal of the protein. Even with these additional sequences the full length transcript is likely to be longer at the 5' end, based on comparison with the *Arabidopsis* protein and the maize genomic sequence.

b) Isolation of and characterisation of maize genes which are orthologs to *Ms41-A*

The *Zm41-A* cDNA was used to screen two different maize

genomic lambda libraries. The first was a commercial library (Clontech, Palo Alto, CA,USA) elaborated with DNA fragments from maize line B73 plantlets. DNA was partially digested with *Mbo*I enzyme and the fragments were cloned into the *Bam*HI site of EMBL-3 (Frischauf *et al*, *J.Mol.Biol.*, **170**:827 (1983)). The insert DNA can be excised from the clone by the enzyme *Sal*I. The second was a lambda library kindly provided by R. Mache (Universite Joseph Fourrier, URA 1178, Grenoble, France) elaborated with DNA fragments from the Mo 17 maize line. DNA was partially digested with the enzyme *Mbo*I and the fragments were cloned into the *Bam*HI site of EMBL-4 (Frischaul *et al*, *supra*). The insert DNA was excised by the enzyme *Eco*RI. The genomic libraries screening was performed following the instructions of Sambrook *et al* (Molecular cloning:a laboratory manual, Cold Spring Harbour Laboratory Press, New York, 1989). 10^6 recombinant Lambda per library were screened and three rounds of screening were performed. Fourteen positive lambda clones were isolated one of which was obtained from the library provided by R. Mache.

DNA from positive lambda clones was extracted and purified using Qiagen columns (Chatsworth, CA, USA) according to the manufacturer's instructions. Then the clones were characterised by Southern analysis (*J.Mol.Biol.*, **98**:503-517 (1975)) in order to establish classes. DNAs from the Clontech library were restricted with *Hind*III and *Eco*RI and double restricted with *Hind*III/*Sal*I. DNAs from the Mache library were restricted with *Hind*III and *Eco*RI and double restricted with *Hind*III /*Eco*RI. DNA fragments were separated on agarose gel, denatured and blotted onto Hybond N$^+$ membrane (Amersham, Buckinghamshire, UK). The blots were hybridised with ^{32}P-

labelled *Zm*41-A cDNA isolated after digestion with *Bam*HI and *Xho*I (the resulting fragment is 2.1 kb long).

Ten lambda clones were different and were distributed in three classes:
class A comprising 5 clones (Z9, Z23, Z27, Z35 and Z36);
class B comprising 4 clones (Z7, Z28, Z29 and Z33); and
class c with only one clone, Z31, isolated from the R. Mache library.

In order to study the sequence of these three classes, the sub-cloning of three different genomic phages (Z31, Z33 and Z35) in the plasmid pBSII SK⁻ (Stratagene, LaJolla, CA, USA) was performed according to the classical cloning method (Sambrook *et al*, *supra*). Hybridizing fragments were firstly slected. After the sequencing of the fragments' extremities with universal primers, oligonucleotides were designed and the sequencing was chieved using the walking primer method.

With the clone Z31, 7.8 kb of continuous sequence data were obtained (see figure 12). To determine the complete gene structure, we have sequenced the entire *Zm*41-A cDNA. This is 2109 bp in length and encodes a putative peptide of 587 amino acids. The comparison between the genomic sequence and the cDNA and 5'RACE sequences indicated that this gene contains at least 12 exons. The insertion reported in the longest RACE products corresponds to the end of intron 4. Thus, the two families of cDNAs might be explained by the presence of two splicing sites in this intron. In the genomic sequence upstream of the end of the RACE products, there was dtected the continuation of the open reading frame of 270bp before an initiation codon at a *Nco*I restriction site. AAsuming that this

initiation site is the right one, the length of the fragment which might contain the promoter sequence was 2.7kb from the *Hind*III site where the sequence starts to the *Nco*I site. Therefore the translation of the *Zm*41-A Z31 gene should give a putative protein of 736 amino acids. The Z31 gene structure is depicted in figure 13.

With the addition of the unspliced sequence (homologous to the end of intron 4) a longer protein might be obtained. Indeed, the longest open reading frame deduced from the genomic sequence Z31 including this insertion sequence exhibits two stop codons in frame. It is also worthwhile noting that there is a clear polymorphism here since the RACE products do not show these stop codons. The mis-splicing phenomenon may be a regulatory mechanism for the expression of the the *Zm*41-A related proteins as has recently been demonstrated in maize for another gene (Burr *et al*, *The Plant Cell*, 8:1249-1259 (1996)). Therefore, either this gene codes for two proteins (736 aa and 131 aa) or it codes for the 736 aa and 772 aa proteins.

Moreover, a slight difference was observed between the *Zm*41-A cDNA and the Z31 genomic sequence in exon ten where a small addition is present (15 bp replaced by 36 bp); this is also in agreement with genetic polymorphism between maize lines. The maize lines used to study the mRNA and the genomic sequence are divergent (A188, B73 and Mo17 respectively). In figure 14 there is provided the alignment of the Z31 protein (736 aa) deduced from the longest open reading frame, with the protein deduced from the *Zm*41-A cDNA (587 aa). We found 15 amino acid changes as well as an additional 7 amino acids for the Z31 protein, these additional amino acids being located

at position 556 of the *Zm*41-A cDNA protein.

For the other two genes, Z33 and Z35, 2.9 Kb and 5.8 Kb were respectively sequenced (see figures 15 and 16). Z35 contains exon 3 in part and the complete exons 4, 5 and 6 from the *Zm*41-A cDNA. Z33 is similar to Z35 but it has a deletion of exon 4 and the 3' end of exon 3. the two have the insertion sequence found in the longest 5' RACE products. In addition, the comparison of the Z33 and Z35 sequences indicates at two deletions in the Z33 gene with respect to the Z35 gene. The first one is 686bp long and starts in the 3' end of exon 3 and extends to the end of exon 4 (with reference to the Z31 gene structure). The latter is located upstream of the sequence homologous to Z31 and the *Zm*41-A cDNA and is 808bp long (see figure 13). Moreover, these two genes differed in their 3' sequenced regions.

Due to the high level of conservation between these 3 sequences it is possible that the Z35 gene derived from Z31 via genetic rearrangements, deletions and/or insertions. Z33 has subsequent deletions from Z35.

Example 9

Genetic mapping of *Zm*41-A loci

58 single seed descent (SSD) maize lines derived from the cross A188 x HD7 (Murigneux *et al*, *Theor.Appl.Genet.*, **87**:278-287 (1993)) were used for genetic mapping by RFLP technology. Hybridisation was performed with radiolabelled *Zm*41-A cDNA (*BamHI-XhoI* fragment, 2.1 Kb) on blots containing DNA from SSD lines and parental lines, digested with *Hind*III or *Eco*RI. Linkage analysis with the other RFLP markers mapped on this population was done using the Mapmaker version 2.0 computer program for

Macintosh (Lander *et al*, *Genomics*, **1**:174-181 (1987)) and map distances were calculated with Kosambi function.

Many polymorphic bands between parental lines were revealed: one or two major bands and a few faint bands. Three loci, named *Zm*41-A.A, *Zm*41-A.B and *Zm*41-A.C were found located on two different chromosomes. *Zm*41-A.A locus corresponding to major bands, was located on the long arm of chromosome 6 at 26 cM from the RFLP marker *umc132* and at 2 cM from the rflp marker *umc62* (Maize Genetics Cooperation Newsletters (MNL) (August 1995) **69**:248). *Zm*41-A.B and *Zm*41-A.C loci, corresponding to faint bands were located on chromosome 2 and were separated from each other by 19 cM. The *Zm*41-A.B locus lies near the centomere between *umc131* (6 cM) and *umc055* (3 cM) markers (MNL, *supra*). The *Zm*41-A.C locus was on the longchromosomic arm between *umc055* (16 cM) and *umc022* (6 cM) (MNL, *supra*). According to the mutant maize genetic map, no obvious male sterile mutant is mapped in those regions. One dominant male sterile mutant, *Ms*21, discovered in 1950 has been assigned on chromosome 6 but not very precisely. This mutation gives sterility only in the presence of the sks1 mutation. Interestingly, this mutation maps on chromosome 2, in the vicinity of the *Zm*41-A.B. Hybridisation on the blots containing DNA from SSD lines, with a Z31 gene specific probe, demonstrated that the Z31 gene corresponds to the *Zm*41-A.A locus on chromosome 6.

CLAIMS:

1. A recombinant or isolated nucleic acid sequence which:

a) encodes the Ms41-A protein from *Arabidopsis*;

b) encodes a Ms41-A like protein;

c) encodes the ms41-A protein from *Arabidopsis*;

d) encodes a ms41-A like protein;

e) comprises a promoter sequence which regulates expression of the Ms41-A protein from *Arabidopsis* or a promoter sequence which regulates expression of a Ms41-A like protein; or

f) hybridises under stringent conditions to Nucleic acid of a), b), c), d) or e) or would do so but for the degeneracy of the genetic code.

2. Nucleic acid as claimed in claim 1 a) wherein the DNA encodes a protein having an amino acid squence as shown in figure 4.

3. Nucleic acid as claimed in claim 1 b) which includes the sequence shown in figures 12, 15 or 16.

4. Nucleic acid as claimed in claim 1 derived from the family *Brassicaceae* or Maize.

5. Nucleic acid as claimed in any one of claims 1 to 4 which comprises a promoter, a coding region and a

transcription termination region.

6. Nucleic acid as claimed in claim 5 having at least a part of the nucleotide sequence shown in figure 3.

7. Nucleic acid as claimed in claim 6 having the nucleotide sequence shown in figure 3 commencing with the base pair labelled 1.

8. Nucleic acid as claimed in claim 1 a), b), c) or d) which includes a promoter sequence which drives expression in a plant tissue involved in the control of fertility.

9. Nucleic acid as claimed in claim 8 wherein the promoter is a tapetum-specific promoter.

10. Nucleic acid as claimed in claim 9 wherein the promoter is the A3, A6 or A9 promoter derived from *Brassicaceae*.

11. Nucleic acid as claimed in claim 1 e) which is operatively coupled to a DNA sequence.

12. Nucleic acid as claimed in claim 11 wherein the DNA sequence encodes a disrupter molecule.

13. Nucleic acid as claimed in claim 12 wherein the disrupter molecule is a lytic enzyme, a ribonuclease, a protease or a lipase.

14. Nucleic acid as claimed in claim 13 wherein the disrupter molecule is a ribonuclease, preferably Barnase.

15. Antisense nucleic acid which includes a transcribable strand of DNA complementary to at least a part of a DNA molecule as defined in any one of claims 1 to 10.

16. Antisense nucleic acid as claimed in claim 15 wherein the antiense nucleic acid is under the control of a constitutive promoter.

17. Antisense nucleic acid as claimed in claim 16 wherein the constisutive promoter is the CaMV35S promoter.

18. Nucleic acid encoding a ribozyme capable of specific cleavage of RNA encoded by a DNA molecule as defined in any one of claims 1 to 10.

19. Nucleic acid as claimed in claim 18 which also includes an appropriate promoter sequence, eg a constitutive promoter.

20. Nucleic acid as claimed in any one of claims 1 to 19 comprising a 3'-transcription regulation signal.

21. Nucleic acid as claimed in any one of claims 1 to 20 which is in the form of a vector.

22. A host cell transformed with nucleic acid as claimed in any one of claims 1 to 21.

23. A process for preparing nucleic acid as claimed in any one of claims 1 to 22, the process comprising coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides.

24. A plant cell including nucleic acid as claimed in any one of claims 1 to 21.

25. A whole plant, or part of a plant, comprising cells as claimed in claim 24.

26. A protein encoded by nucleic acid as defined in claim 1 a), b), c) or d).

27. A protein as claimed in claim 26 which has the amino acid sequence shown in figure 4.

28. The use of nucleic acid as defined in any one of claims 1 to 21 in the preparation of a transgenic plant.

29. A method for the production of a transgenic plant which comprises the step of transforming plant propagating material with nucleic acid as defined in any one of claims 1 to 21.

The transposed 35S *Ac* element is linked to the male sterile mutation 41-A

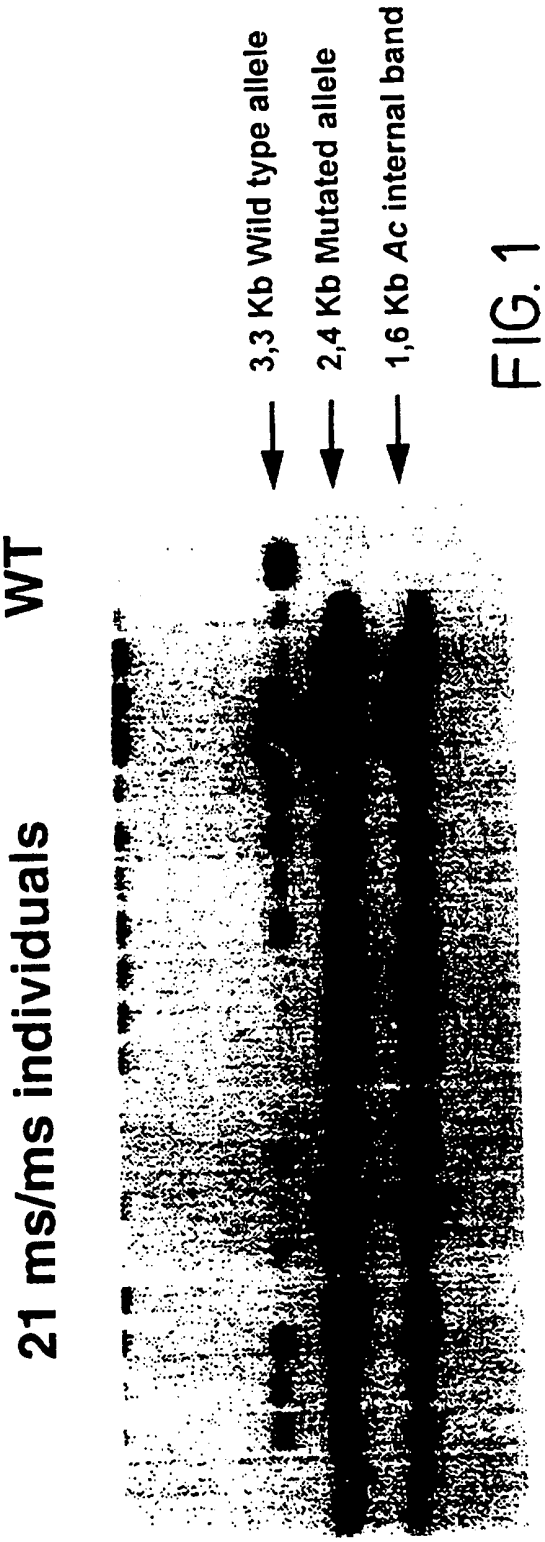

FIG. 1

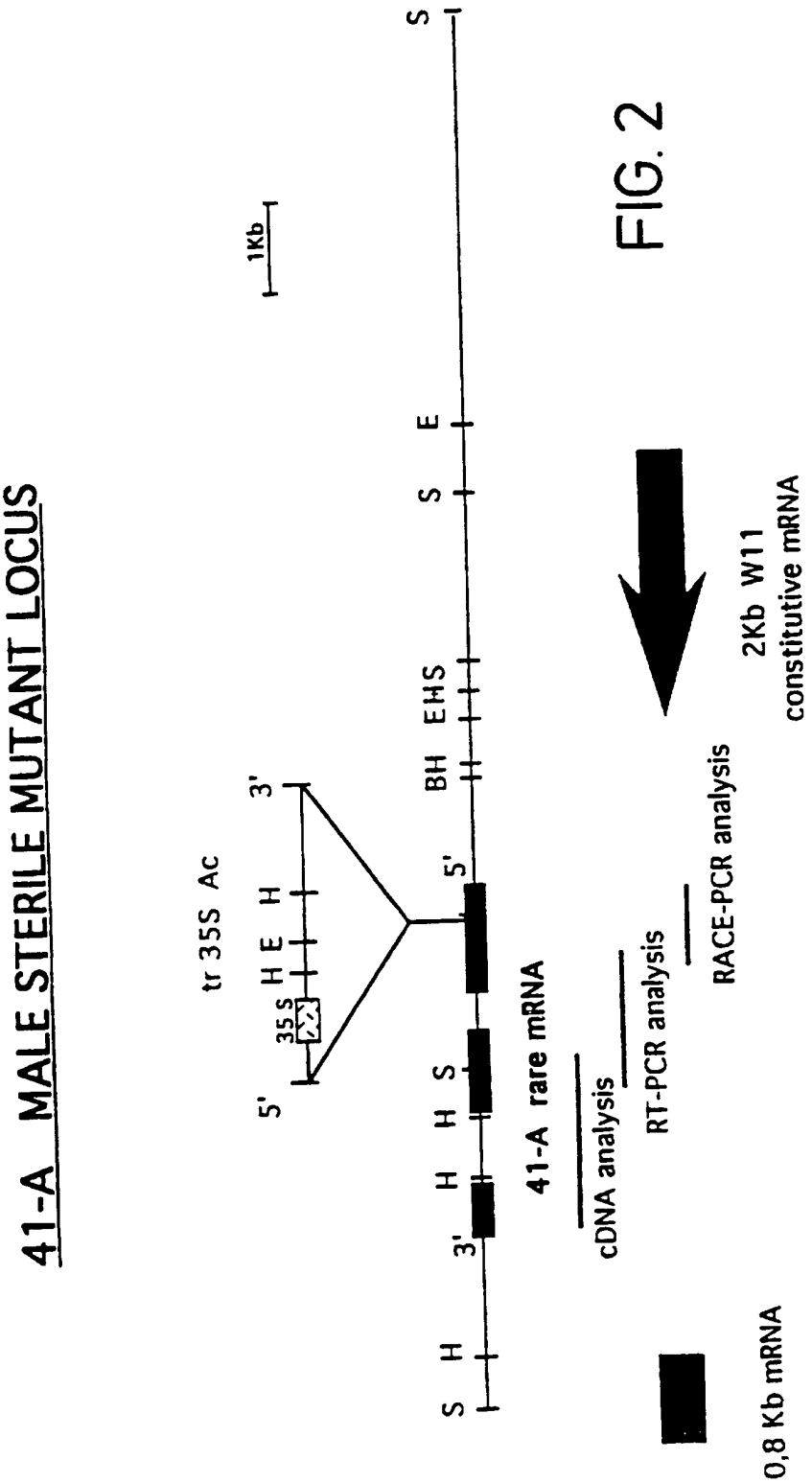
41-A MALE STERILE MUTANT LOCUS
1Kb
S
S E
BH EHS
5'
3'
tr 35S Ac
35S
H E H
5'
H H S
3'
S H
41-A rare mRNA
cDNA analysis
RT-PCR analysis
RACE-PCR analysis
2Kb W11
constitutive mRNA
0,8 Kb mRNA

FIG. 2

FIG. 3(I)

EcoRI

TCCTAACTTTCTTTGCGGCATTTCTTATAATACTTCGTCAGTTTTCAGAATTCTTAAATC
-1780 -1760 -1741

TTTTTGCTGTGTTCTTATAAAGAAACATCATCTATTAAAGTTGTCTTCGTTTGGATTTGG
-1720 -1700 -1681

TTTTGATGACTTTGGGAAATATTTATGTTTAAGAAGGTTTCATTGGTCATTGACTTTTAT
-1660 -1640 -1621

ATATTATATCGTAACCATGATGTGATAGTGGGCCTTAGATCAACAAACATGCGAAAAACA
-1600 -1580 -1561

GAAGCAGAGGCCCGTTTCAACGGAGCATAATAAATTGCATTCTCTGTCTTTTGTTTTTAG
-1540 -1520 -1501

GTTTTTTTTTTAACTGATAGATGTGCCGTCGAAAATAATATTGATATTTAAAAATTCACA
-1480 -1460 -1441

ACAAACATTCTTAACTGACCCACCCATCTATCTGCTATTCCCACGCGCCAAGGAAAATAA
-1420 -1400 -1381

TAATAATAGCGAAATTGATTTTACATTTATTTATTGATAGATAATTTGTGTATTGTTAAG
-1360 -1340 -1321

ATTAACAGATTTTAAGGGATTAAAGTGGAAAAGGTAAACCGAAGACAACTTGCCATTTAC
-1300 -1280 -1261

TGATTTACAACAATCCAAATTTAAAAACAAATGGTCCCAGTTTTTAGGGTTGTCACTTAA
-1240 -1220 -1201

ATTTATCGAAATATTTACACTTTAATTGGGTAAAACATAATGGACAGAAAAACAAATATT
-1180 -1160 -1141

HindIII

GTGACAAACAAAAAAACATGTTTTCACCAAGAAAAACAAAAACAAAAAAGATGTAAAGCT
-1120 -1100 -1081

TTTCTTACATCTGTACAAAATAAAAGCAGACGAAATTGTACTTTATTTTCCTTATTAAAT
-1060 -1040 -1021

TGTCGGTATGTTTTATATGTTGTGAAAAGTAGAATGGATAACCAAATAAAAATTACTGCA
-1000 -980 -961

HindIII

TCTTAATAAAGTTGGTTCAACCGGTTTAAAATGTATTTTTTTAGTGTTAACAACTTAAAG
-940 -920 -901

CTTTTTTCGATTATCGAATTGCAACAAACAAATATATTAACAGAAAAAAGGAATCATGTA
-880 -860 -841

TCTATTTCAATATCCTGTTTTTTTTCTTCCATTTGGATATTTAGATCTTTTTCTGAATTT
-820 -800 -781

ATCTTGTTCTTAAATTAAAACAGAAAAAAAGATTAAAAGTAAGACAGCTTGCTAATGGCA
-760 -740 -721

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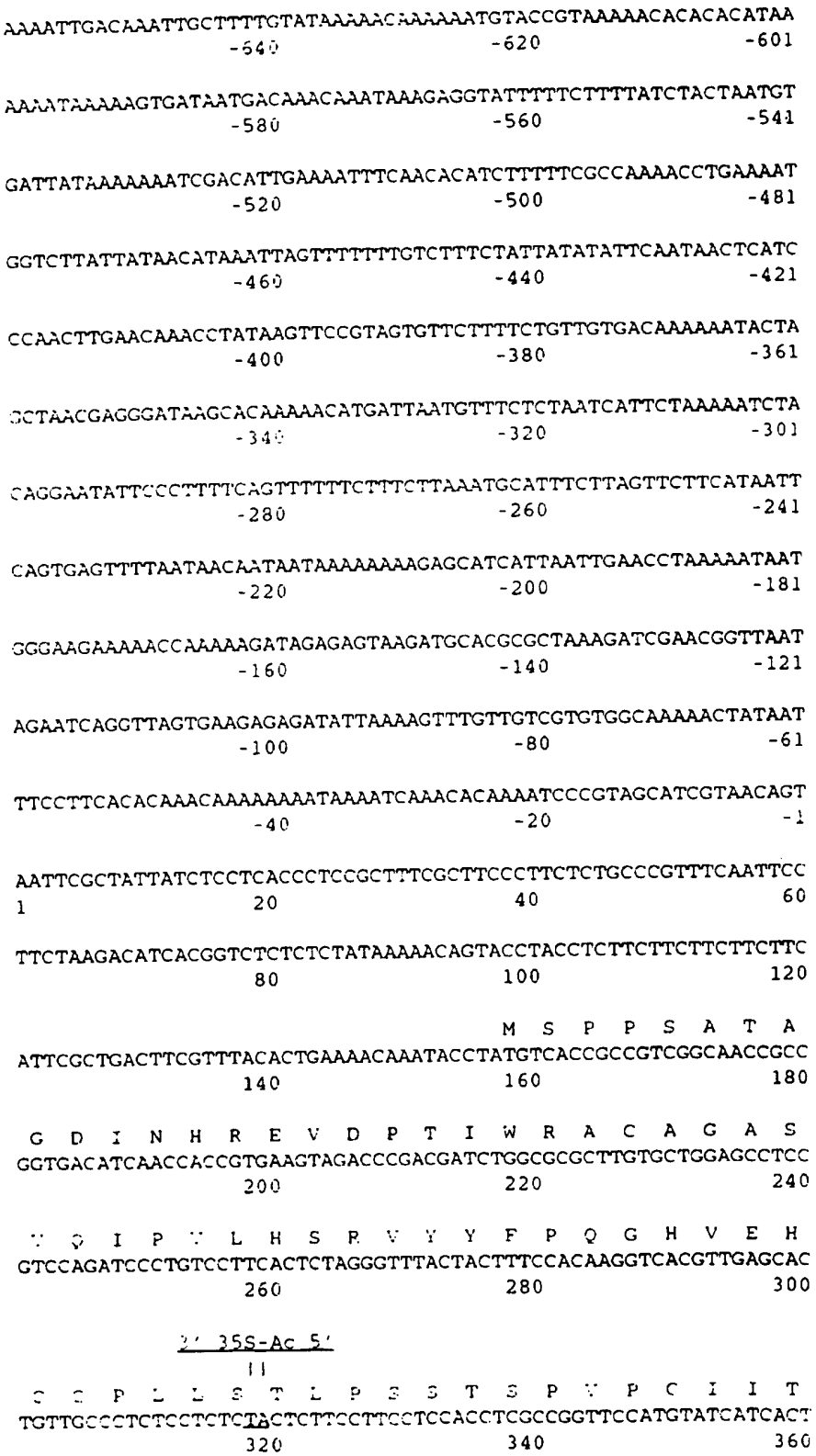
-700 -680 -661

FIG. 3(II)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-580 -560 -541
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-520 -500 -481
GGTCTTATTATAACATAAATTAGTTTTTTTGTCTTTCTATTATATATTCAATAACTCATC
-460 -440 -421
CCAACTTGAACAAACCTATAAGTTCCGTAGTGTTCTTTTCTGTTGTGACAAAAAATACTA
-400 -380 -361
GCTAACGAGGGATAAGCACAAAAACATGATTAATGTTTCTCTAATCATTCTAAAAATCTA
-340 -320 -301
CAGGAATATTCCCTTTTCAGTTTTTTCTTTCTTAAATGCATTTCTTAGTTCTTCATAATT
-280 -260 -241
CAGTGAGTTTTAATAACAATAATAAAAAAAAAGAGCATCATTAATTGAACCTAAAAATAAT
-220 -200 -181
GGGAAGAAAAACCAAAAAGATAGAGAGTAAGATGCACGCGCTAAAGATCGAACGGTTAAT
-160 -140 -121
AGAATCAGGTTAGTGAAGAGAGATATTAAAAGTTTGTTGTCGTGTGGCAAAAACTATAAT
-100 -80 -61
TTCCTTCACACAAACAAAAAAAAATAAAATCAAACACAAAATCCCGTAGCATCGTAACAGT
-40 -20 -1
AATTCGCTATTATCTCCTCACCCTCCGCTTTCGCTTCCCTTCTCTGCCCGTTTCAATTCC
1 20 40 60
TTCTAAGACATCACGGTCTCTCTCTATAAAAACAGTACCTACCTCTTCTTCTTCTTCTTC
80 100 120
M S P P S A T A
ATTCGCTGACTTCGTTTACACTGAAAACAAATACCTATGTCACCGCCGTCGGCAACCGCC
140 160 180
G D I N H R E V D P T I W R A C A G A S
GGTGACATCAACCACCGTGAAGTAGACCCGACGATCTGGCGCGCTTGTGCTGGAGCCTCC
200 220 240
V Q I P V L H S R V Y Y F P Q G H V E H
GTCCAGATCCCTGTCCTTCACTCTAGGGTTTACTACTTTCCACAAGGTCACGTTGAGCAC
260 280 300
2' 35S-Ac 5'
C C P L L S T L P S S T S P V P C I I T
TGTTGCCCTCTCCTCTCTACTCTTCCTTCCTCCACCTCGCCGGTTCCATGTATCATCACT
320 340 360

FIG. 3(III)

S I Q L L A D P V T D E V F A H L I L Q
TCAATCCAGTTGCTCGCCGATCCGGTTACCGACGAGGTCTTTGCTCACCTTATTCTTCAA
380 400 420

P I T Q Q Q F T P T N Y S R F G R F D G
CCGATCACGCAGCAGCAGTTTACTCCGACTAATTATTCACGATTCGGCAGATTCGATGGC
440 460 480

D V D D N N K V T T F A K I L T P S D A
GATGTTGATGATAACAACAAGGTGACTACCTTCGCCAAAATTCTCACGCCTTCTGATGCT
500 520 540

N N G G G F S V P R F C A D S V F P L L
AACAATGGAGGTGGCTTCTCCGTTCCTCGTTTCTGTGCTGATTCCGTCTTCCCTCTGCTT
560 580 600

N F Q I D P P V Q K L Y V T D I H G A V
AATTTTCAAATCGATCCACCGGTTCAGAAGCTCTACGTCACTGATATCCATGGAGCTGTT
620 640 660

W D F R H I Y R G T P R R H L L T T G W
TGGGATTTCAGGCATATCTATCGCGGTACACCGAGGCGTCACTTGCTAACAACGGGATGG
680 700 720

S K F V N S K K L I A G D S V V F M R K
AGTAAGTTTGTCAATAGCAAGAAGCTCATCGCTGGAGATTCGGTTGTGTTTATGAGAAAA
740 760 780

S A D E M Y I G V R R T P I S S S D G G
TCTGCAGATGAGATGTACATCGGTGTTAGGCGAACTCCGATCTCAAGCAGCGACGGAGGA
800 820 840

S S Y Y G G D E Y N G Y Y S Q S S V A K
AGTAGCTATTACGGAGGAGATGAGTATAACGGTTACTACAGTCAGAGTAGCGTTGCCAAG
860 880 900

E D D G S P K K T F R R S G N G K L T A
GAAGATGATGGGAGTCCGAAGAAGACGTTTAGGAGATCTGGGAATGGTAAGTTGACTGCT
920 940 960

E A V R S I N R A S Q G L P F E V V F Y
GAGGCTGTACGATCGATCAATAGAGCGTCTCAGGGATTACCGTTTGAGGTGGTGTTTTAT
980 1000 1020

P A A G W S E F V V R A E D V E S S M S
CCGGCTGCTGGATGGTCTGAGTTTGTTGTGAGAGCTGAAGATGTTGAGTCTTCAATGTCT
1040 1060 1080

M Y W T P G T R V K M A M E T E D S S R
ATGTATTGGACTCCTGGGACTCGAGTCAAGATGGCTATGGAGACTGAAGATTCTTCTCGG
1100 1120 1140

I T W F Q G I V S S T Y Q E T G P W R G
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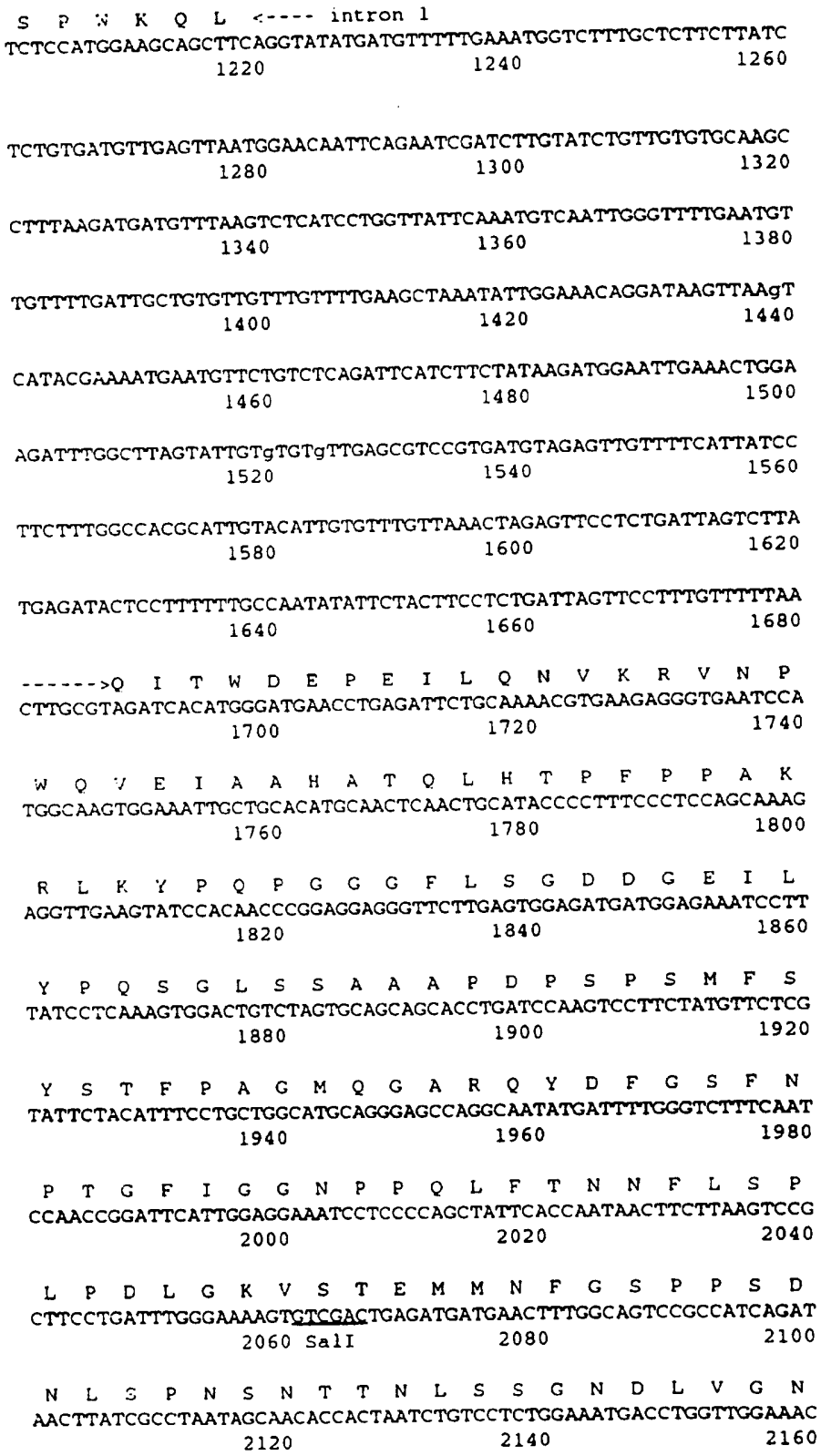
1160 1180 1200

FIG. 3(IV)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1280 1300 1320
CTTTAAGATGATGTTTAAGTCTCATCCTGGTTATTCAAATGTCAATTGGGTTTTGAATGT
1340 1360 1380
TGTTTTGATTGCTGTGTTGTTTGTTTTGAAGCTAAATATTGGAAACAGGATAAGTTAAgT
1400 1420 1440
CATACGAAAATGAATGTTCTGTCTCAGATTCATCTTCTATAAGATGGAATTGAAACTGGA
1460 1480 1500
AGATTTGGCTTAGTATTGTgTGTgTTGAGCGTCCGTGATGTAGAGTTGTTTTCATTATCC
1520 1540 1560
TTCTTTGGCCACGCATTGTACATTGTGTTTGTTAAACTAGAGTTCCTCTGATTAGTCTTA
1580 1600 1620
TGAGATACTCCTTTTTTGCCAATATATTCTACTTCCTCTGATTAGTTCCTTTGTTTTTAA
1640 1660 1680
------>Q I T W D E P E I L Q N V K R V N P
CTTGCGTAGATCACATGGGATGAACCTGAGATTCTGCAAAACGTGAAGAGGGTGAATCCA
1700 1720 1740
W Q V E I A A H A T Q L H T P F P P A K
TGGCAAGTGGAAATTGCTGCACATGCAACTCAACTGCATACCCCTTTCCCTCCAGCAAAG
1760 1780 1800
R L K Y P Q P G G G F L S G D D G E I L
AGGTTGAAGTATCCACAACCCGGAGGAGGGTTCTTGAGTGGAGATGATGGAGAAATCCTT
1820 1840 1860
Y P Q S G L S S A A A P D P S P S M F S
TATCCTCAAAGTGGACTGTCTAGTGCAGCAGCACCTGATCCAAGTCCTTCTATGTTCTCG
1880 1900 1920
Y S T F P A G M Q G A R Q Y D F G S F N
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1940 1960 1980
P T G F I G G N P P Q L F T N N F L S P
CCAACCGGATTCATTGGAGGAAATCCTCCCCAGCTATTCACCAATAACTTCTTAAGTCCG
2000 2020 2040
L P D L G K V S T E M M N F G S P P S D
CTTCCTGATTTGGGAAAAGTGTCGACTGAGATGATGAACTTTGGCAGTCCGCCATCAGAT
2060 SalI 2080 2100
N L S P N S N T T N L S S G N D L V G N
AACTTATCGCCTAATAGCAACACCACTAATCTGTCCTCTGGAAATGACCTGGTTGGAAAC
2120 2140 2160

FIG. 3(V)

```
 R  G  P  L  S  K  K  V  N  S  I  Q  L  F  G  K  I  I  T  V
CGAGGCCCCCTTTCAAAGAAAGTTAACTCGATTCAGTTGTTTGGCAAGATCATTACCGTG
                2180                2200                2220

 E  E  H  S  E  S  G  P  A  E  S  G  L  C  E  E  D  G  S  K
GAGGAGCATTCTGAGAGCGGTCCTGCAGAGTCTGGCTTGTGTGAAGAGGATGGCAGCAAA
                2240                2260                2280

 E  S  S  D  N  E  T  Q  L  S  L  S  H  A  P  P  S  V  P  K
GAGTCCAGCGACAATGAGACACAGTTGTCCTTATCACATGCTCCTCCAAGCGTGCCTAAA
                2300                2320                2340

 H  S  N  S  N  A  G  S  S  S  Q<------ intron 2
CATTCCAACAGCAACGCAGGTTCTAGCTCCCAAGGTATATTCCGATCTCTCTCAAGTACA
                2360                2380                2400
                        HindIII
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                2420                2440                2460

TTCCTTTCCCATGAACTATATTATGTAGAGTAGGAAACACAATCATGATTTCTGATATGA
                2480                2500                2520

CTTGACTGATGATGATACTTGTgAAAACTATCTATATATCTCTTCAGTAATCAGTCGCCT
                2540                2560                2580

TGAGGTAATTGGAATTTGGAACTTGAACATTACTTGGATTTTAACTTTTCAATAGCATAA
                2600                2620                2640

GCNTTCCTGTTTCATCATATATGTTTCACTATACTTGTATGCTTTTATTACTGCTGATAT
                2660                2680                2700

TTACTATTCCTGCTATTTTTTTTGGGTCTCGTTAACGGTAATAAGGACACAGAATTGGCT
                2720                2740                2760

CTTTTATCCATCAGAACTAGACATTACTGTACAAGTAGATGAAGAATTATGTGGTTCCAT
                2780                2800                2820
                   HindIII
TACAAATTTAATTTGCAGAAAGCTTGAAGCTGCTGCTTATAGACGATTATAATGTTGGAA
                2840                2860                2880

       HindIII                                      -------> G  *
GATCCTGAAGCTTGGAATGATTTGTACTTTTCTTTTGTTTGTGTGTGTTTTGACAGGTTA
                2900                2920                2940

AAAAGTGAAAGAAGTGGTGGATCTTTGCTGGAATCTCCAAGTCCTAAGTAGTAGTAGTAG
                2960                2980                3000

TACATTATATATAATTCTGTTGTTTCTGCAATTGACTTTTCTCTGGCTTTTCTTTGCCAC
                3020                3040                3060

GTGACGATTCCGGTTTTTACTTTCTTTCTTTTTTTTTTTATCAATTTCTCAGACACATTTG
                3080                3100                3120

ATGAACATCTCGCTCTCATCTAATCGTTAACTATTTTTATTGGGGTAAATGTCTGGATTT
                3140                3160                3180

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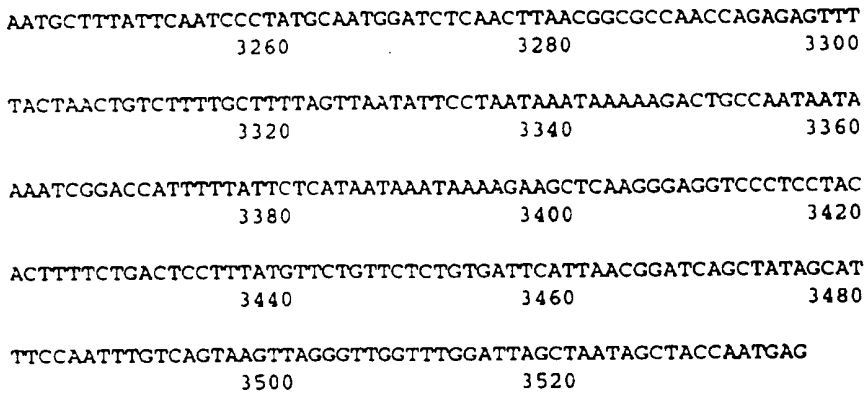
FIG. 3(VI)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3280
3300
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3320
3340
3360
AAATCGGACCATTTTTATTCTCATAATAAATAAAAGAAGCTCAAGGGAGGTCCCTCCTAC
3380
3400
3420
ACTTTTCTGACTCCTTTATGTTCTGTTCTCTGTGATTCATTAACGGATCAGCTATAGCAT
3440
3460
3480
TTCCAATTTGTCAGTAAGTTAGGGTTGGTTTGGATTAGCTAATAGCTACCAATGAG
3500
3520

Sequence Range: 1 to 584

```
                                                                  50
                                                                   *
    MSPPSATAGD  INHREVDPTI  WRACAGASVQ  IPVLHSRVYY  FPQGHVEHCC
                                                                 100
                                                                   *
    PLLSTLPSST  SPVPCIITSI  QLLADPVTDE  VFAHLILQPI  TQQQFTPTNY
                                                                 150
                                                                   *
    SRFGRFDGDV  DDNNKVTTFA  KILTPSDANN  GGGFSVPRFC  ADSVFPLLNF
                                                                 200
                                                                   *
    QIDPPVQKLY  VTDIHGAVWD  FRHIYRGTPR  RHLLTTGWSK  FVNSKKLIAG
                                                                 250
                                                                   *
    DSVVFMRKSA  DEMYIGVRRT  PISSSDGGSS  YYGGDEYNGY  YSQSSVAKED
                                                                 300
                                                                   *
    DGSPKKTFRR  SGNGKLTAEA  VRSINRASQG  LPFEVVFYPA  AGWSEFVVRA
                                                                 350
                                                                   *
    EDVESSMSMY  WTPGTRVKMA  METEDSSRIT  WFQGIVSSTY  QETGPWRGSP
                                                                 400
                                                                   *
    WKQLQITWDE  PEILQNVKRV  NPWQVEIAAH  ATQLHTPFPP  AKRLKYPQPG
                                                                 450
                                                                   *
    GGFLSGDDGE  ILYPQSGLSS  AAAPDPSPSM  FSYSTFPAGM  QGARQYDFGS
                                                                 500
                                                                   *
    FNPTGFIGGN  PPQLFTNNFL  SPLPDLGKVS  TEMMNFGSPP  SDNLSPNSNT
                                                                 550
                                                                   *
    TNLSSGNDLV  GNRGPLSKKV  NSIQLFGKII  TVEEHSESGP  AESGLCEEDG


    SKESSDNETQ  LSLSHAPPSV  PKHSNSNAGS  SSQG
```

FIG. 4

FIG. 5

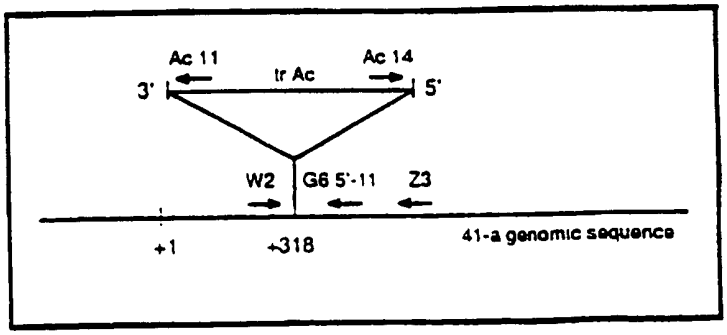
LIST OF PRIMERS

Ac 11	5' CGTATCGGTTTTCGATTACCGTATT 3' located at position 4419-4443 on Ac sequence	25-mer
Ac 14	5' CGTTTCCGTTTCCGTTTACCGTTTT 3' located at position 145-127 on Ac sequence	25-mer
W2	5' TGCTTGTGCTGGAGCC 3' located at position 221-237 on 41-a genomic sequence Concentration : 5296 ng / ul 1.0633 nmoles / ul	19-mer
Z3	5' GTTATCATCAACATCGCCATCGAATCTGCCG located at position 495-465 on 41-a genomic sequence Concentration : 13811 ng / ul 1.4555 nmoles / ul	31-mer
G6 5'-11	5' CTGCTGCTGCGTGATCGG located at position 438-421 on 41-a sequence Concentration : 4943 ng / ul 0.8828 nmoles / ul	18-mer

COMMENTS :

Lenght of amplification product

W2 / G6 5'-11	217 bp
W2 / Ac 11	240 bp
Ac 14 / G6 5'-11	265 bp



Wild type Sequence	Cys Pro TGC CCT		Leu Leu Ser Thr Leu CTC CTC TCT ACT CTT	Alleles
Female A or B	TGC CCT	CTC CTC TC (3' Ac 5')	C TCC TCT C TA CTC TT	ms 41:: 35S Ac
	TGC CCT	CTC CTC AG	T CCT CTC TAC TCT T	ms 41-1(+7 bp)
Male parent	TGC CCT	CTC CTC TC (3' Ac 5')	C TCC TCT C TA CTC TT	ms 41:: 35S Ac
	TGC CCT		CTC CTC TC T ACT CTT	Ms 41
Revertant H	TGC CCT		CTC CTC TC T ACT CTT	Ms 41-R
	TGC CCT	CTC CT	C TCC TCT C TA CTC TT	ms 41-2 (+5 bp)
Revertant K	TGC CCT		CTC CTC TC T ACT CTT	M s 41-R
	TGC CCT	CTC C AG	TCC TCT C TA CTC TT	ms 41-3 (+5 bp)
Revertant F	TGC CCT		CTC CTC TC T ACT CTT	Ms 41-R
	TGC CCT	CTC CTC T G	T CCT CTC TAC TCT T	ms 41-5 (+7 bp)
Revertant C	TGC CCT		CTC CTC TC T ACT CTT	Ms 41-R
	TGC CCT	CTC CTC T GA G	TC CTC TC T ACT CTT	ms 41-4 (+9bp)
Revertant M	TGC CCT	CTC	CTC CTC TC T ACT CTT	Ms 41-1R (+3 bp)
	TGC CCT	CTC CTC AG	T CCT CTC T ACT CTT	ms 41-1 (+7 bp)
Revertant A	TGC CCT	CTC	CTC CTC TC T ACT CTT	Ms 41-1R (+3 bp)
	TGC CCT	CTC CTC TC (3' Ac 5')	C TCC TCT C TA CTC TT	ms 41:: 35S Ac
Revertant L	TGC CCT	CTC CTC G	T CCT CTC TAC TCT T	Ms 41-2R (+6 bp)
	TGC CCT	CTC CTC TC (3' Ac 5')	C TCC TCT C TA CTC TT	ms 41:: 35S Ac

Footprints and alleles induced by 35SAc excision from the ms 41-a locus

FIG. 6

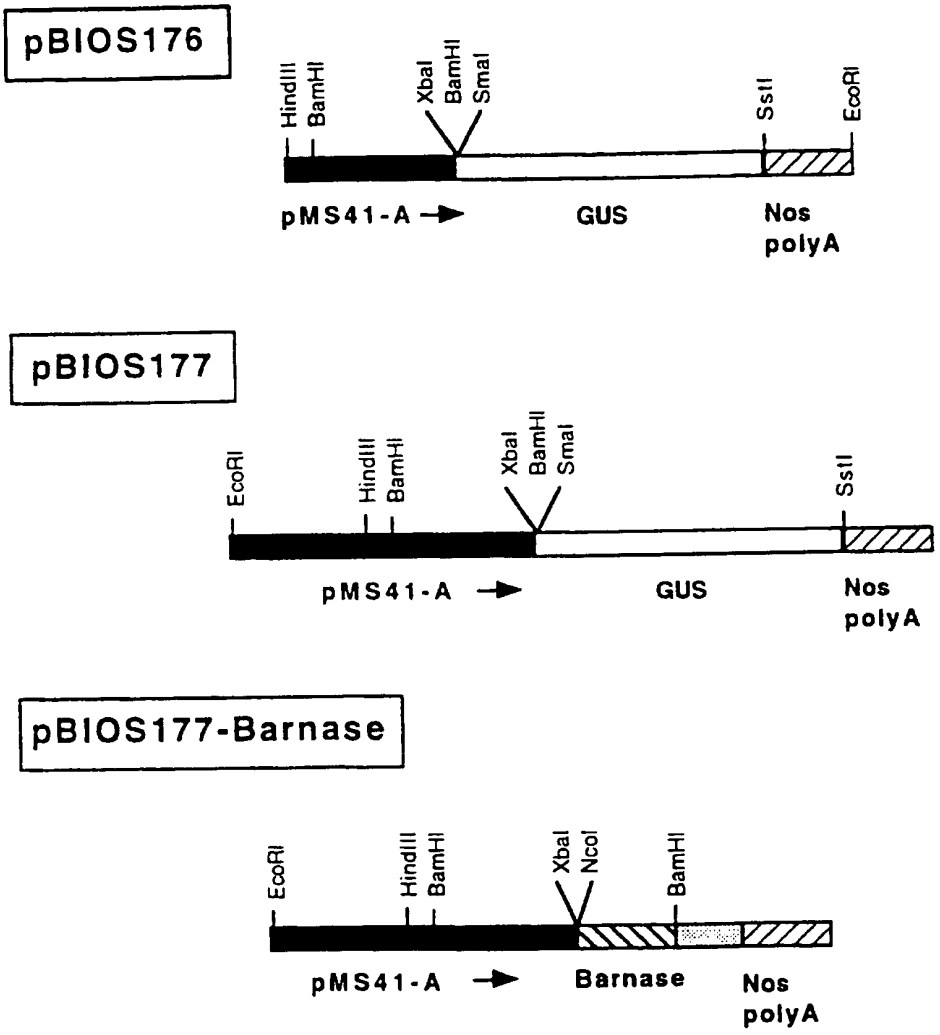
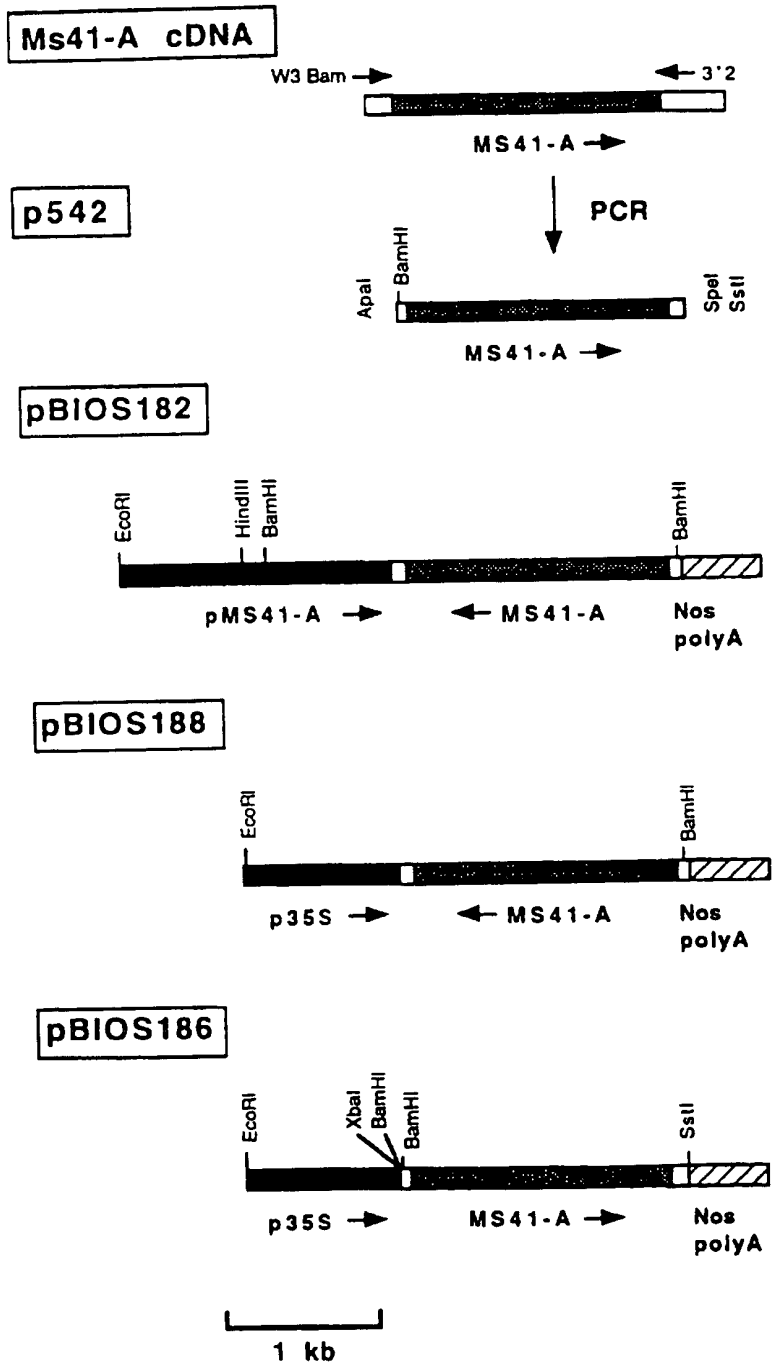

pBIOS176
HindIII
BamHI
XbaI
BamHI
SmaI
SstI
EcoRI
pMS41-A
GUS
Nos
polyA
pBIOS177
EcoRI
HindIII
BamHI
XbaI
BamHI
SmaI
SstI
pMS41-A
GUS
Nos
polyA
pBIOS177-Barnase
EcoRI
HindIII
BamHI
XbaI
NcoI
BamHI
pMS41-A
Barnase
Nos
polyA

FIG. 7

FIG. 8


Ms41-A cDNA
W3 Bam
3'2
MS41-A
p542
PCR
ApaI
BamHI
SpeI
SstI
MS41-A
pBIOS182
EcoRI
HindIII
BamHI
BamHI
pMS41-A
MS41-A
Nos polyA
pBIOS188
EcoRI
BamHI
p35S
MS41-A
Nos polyA
pBIOS186
EcoRI
XbaI
BamHI
BamHI
SstI
p35S
MS41-A
Nos polyA
1 kb

Clustal Alignment of 41a related sequences

```
ZmVP1    515  ..........................................LLQKVLKQSDVGS
OSR1187    1  ..........................................EKRLTPSDVGK
ATTS3975   4  LRKHTYNEELEQSKRRRNGNGNMTRTLLTSGLSNDGVSTTGFRSAEALFEKAVTPSDVGK
At41a     73  LADPVTDEVFAHLILQPITQQQFTPTNYSRFGRFDGDVDDNNKVTT--FAKILTPSDANN
OSS2204    1  ..............................AVKRLARIPHMFCKTLTASDTST
Zm41a      1  ........................DGSAEDGVRKGETVKQRFSRMPHMFCKTLTASDTST
```

```
|ZmVP1      LGRIVLPKKEAEVHLP----ELKTRDGISIPMEDIGTSRVWNMRYRFWPNNKSRMYLLEN|
|OSR1187    LNRLVIPKQXAERYFXLGGGDSGX-KXLLLSXEDES-GKPWRFRYSYWTSSQS--YVLXK|
|ATTS3975   LNRLVIPKHHAEKHFPLPSSNVSV-KGVLLNFEDVN-GKVWRFRYSYWNSSQS--YVLTK|
|T21748     LNRLVIPKQHAEKHFPLPSPSPAVTKGVLINFEDVN-RKVWRFRYSYWNSSQS--YVLTK|
|At41a      GGGFSVPRFCADSVFPLL--NFQIDPPVQKLYVTDIHGAVWDFRHIYR--GTPRRHLLTT|
|OSS2204    HGGFSVPRRAAEDCFPPL--DYSLQRPFQELVAKDLHGTEWRFRHIYR--GQPRRHLLTT|
|Zm41a      HGGFSVPRRAAEDCFPPL--DYSQQRPSQELVAKDLHGTEWRFRHIYR--GQPRRHLLTT|
|
```

```
ZmVP1          T-GEFVRSNELQEGD
ATTS3975       GWSRFVKEKNLRAGD
T21748         GWSRFVKEKNLRAGN
ATTS1074   1   ..........................................GFSGFLRDDESTTTTSKLM
At41a          GWSKFVNSKKLIAGDSVVFMRKSADEMYI-GVRRTPISSSDGGSSYYGGDEYNGYYSQSS
OSS2204        GWSGFINKKKLVSGDCSAIPQEVKMENFDWGVRRAA-QLKNAISF
Zm41a          GWSAFVNKKKLVSGD
```

```
ATTS1074       MMKRNGNNDGNA---AATGRVRVEAVAEAVARAACGQAFEVVYYPRASTPEFCVKAADVR
At41a          VAKEDDGSPKKTFRRSGNGKLTAEAV-RSINRASQGLPFEVVFYPAAGWSEFVVRAEDVE
```

```
ATTS1074       SAMRIRWCSGMRFKMAFETEDSSRISWFMGTVSAVQVADPIRWPNSPWRLLQVAWDEPDL
At41a          SSMSMYWTPGTRVKMAMETEDSSRITWFQGIVSST-YQETGPWRGSPWKQLQITWDEPEI
```

```
At41a          LQNVKRVNPWQVEIAAHATQLH-TPFPPAKRLKYPQP------------GGGFLSGDDG
ATTS1074       LQNVKRVSPWLVELVSNMPTIHLSPFSPRKKIRIPQPFEFPFHGTKFPIFSPGFANNGGG
```

```
At41a          EILY--------PQSGLSSAAAPD-----PSPSMFS---YSTFPAG--MQGARQYDFGSF
ATTS1074       ESMCYLSNDNNNAPEGIQGARQAQQLFGSPSPSLLSDLNLSSYTGNNKLHSPAMF-LSSF
```

```
At41a          NPT----------------GFIGGNPPQ----------------LFTNNFLSPLPDLG
ATTS1074       NPRHHHYQARDSENSNNISCSLTMGNPAMVQDKKKSVGSVKTHQFVLFGQPILTEQQVMN
```

```
At41a          KVSTEMMNFGSPPSDNLSPNSNT-----TNLSSGN-------DLVGNRGPLSKKVNSIQL
ATTS1074       RKRFLEEEAEAEEEKGLVARGLTWNYSLQGLETGHCKVFMESEDVGRTLDLSVIGSYQEL
```

```
At41a          FGKI---ITVEEHSE-----SGPAESGLCEEDGSKESSD-----NETQLSLSHAPPSVPK
ATTS1074       YRKLAEMFHIEERSDLLTHVVYRDANGVIKRIGDEPFSDFMKATKRLPIKMDIGGDNVRK
```

```
At41a          HSNSNAGSSSQG--------------
ATTS1074       TWITGIRTGENGIDASTKTGPLSIFA
```

FIG. 9

Zm41a 5 prime DNA sequence and proposed ORF

```
    AGGACGGCAGCGCCGAGGACGGCGTACGGAAGGGGGAAACCGTGAAGCAGCGGTTCTCGC
  1 ---------+---------+---------+---------+---------+---------+ 60
    TCCTGCCGTCGCGGCTCCTGCCGCATGCCTTCCCCCTTTGGCACTTCGTCGCCAAGAGCG
      D  G  S  A  E  D  G  V  R  K  G  E  T  V  K  Q  R  F  S  R

    GGATGCCGCACATGTTCTGCAAGACGCTCACGGCCTCCGACACCAGCACGCACGGGGGTT
 61 ---------+---------+---------+---------+---------+---------+ 120
    CCTACGGCGTGTACAAGACGTTCTGCGAGTGCCGGAGGCTGTGGTCGTGCGTGCCCCCAA
      M  P  H  M  F  C  K  T  L  T  A  S  D  T  S  T  H  G  G  F

    TCTCCGTGCCGCGCCGCGCCGCCGAGGACTGCTTCCCGCCTCTGGACTACAGCCAGCAGC
121 ---------+---------+---------+---------+---------+---------+ 180
    AGAGGCACGGCGCGGCGCGGCGGCTCCTGACGAAGGGCGGAGACCTGATGTCGGTCGTCG
      S  V  P  R  R  A  A  E  D  C  F  P  P  L  D  Y  S  Q  Q  R

    GACCGTCGCAGGAGCTTGTGGCCAAGGATTTGCACGGAACCGAGTGGAGGTTCCGCCACA
181 ---------+---------+---------+---------+---------+---------+ 240
    CTGGCAGCGTCCTCGAACACCGGTTCCTAAACGTGCCTTGGCTCACCTCCAAGGCGGTGT
      P  S  Q  E  L  V  A  K  D  L  H  G  T  E  W  R  F  R  H  I

    TTTATCGAGGGCAGCCCCGCAGACACCTTTTAACCACTGGATGGAGTGCCTTTGTCAACA
241 ---------+---------+---------+---------+---------+---------+ 300
    AAATAGCTCCCGTCGGGGCGTCTGTGGAAAATTGGTGACCTACCTCACGGAAACAGTTGT
      Y  R  G  Q  P  R  R  H  L  L  T  T  G  W  S  A  F  V  N  K

    AGAAGAAGCTTGTCTCAGGGGAC
301 ---------+---------+---  323
    TCTTCTTCGAACAGAGTCCCCTG
      K  K  L  V  S  G  D
```

FIG. 10

FIG. 11

Dendrogram based on the clustal alignment of 41a related sequences

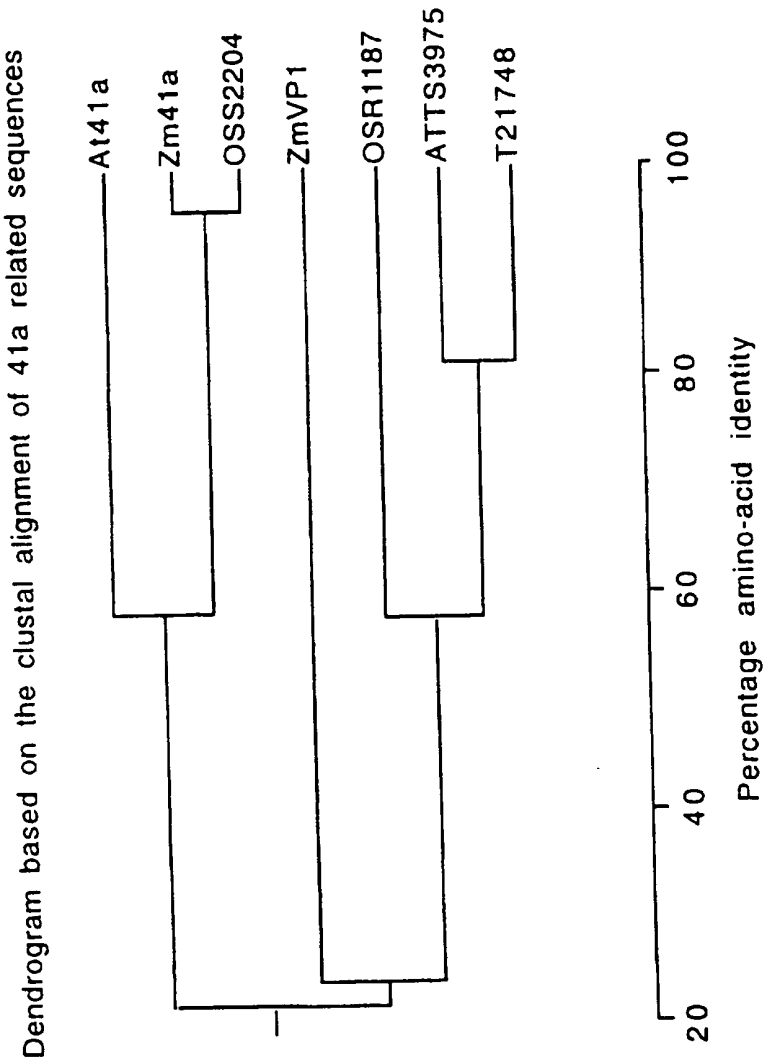


FIG. 12(I)

70
*
aagctttagt gactagtgag agtgatttgt tgtgttcttt tgagctcttg cgcttggatt gctttcttct

140
*
ttctcattct ttcttgagat caatactcac ttgtaaccga ggcaagagac accaattgtg tggtggtcct

210
*
tgcgggtaag ttttgttccc ggttgatttg agaagagaaa gctcactcgg tccgagggac cgtttgaaag

280
*
agggaagggg ttgaaaaaga cccggccttt gtggcctcct caatggggag taggtttgcg aaaaccgaac

350
*
ctcggtaaaa caaatccgcg tgtcacactt cttatctgct tgcgatttgt ttttcaccct ctctcgcgga

420
*
ctcgattata tttctaacgc taacccgact tgtagttgtg attaactttg taaatttcag tttcgcccta

490
*
ttcacccccc tctatgcgac tttcagtagt tcatctatcc catgttttac ccctatttgc ttggatctga

560
*
gctgattgcg acttagagac taaactgctg aacttatgaa cctgtgaata aaatactaag taaactagtt

630
*
agtccgaatg tttgtgatag tcatcaagca ccaaaatcaa tataaaaatg gtttaaggcc aatttccttt

700
*
cgcaaagata tggaatgtca taacccgtca atccttcatg taacaatggt cgtgcgttcc ctcaaccata

770
*
caaagggaca tggccgcact gaaaaggcag acacacatag ttttacatat tttctacgct agcacaatag

840
*
cctcgttctc cactctgcaa ctcacgaaaa cagtaacaaa aacttcaaca acatactagg catattttct

910
*
ctccaaactg gtctaaaaac tctcttcaaa ctcacttcga gcaaggtaat cgggacatta gcaccgcaat

980
*
ccctttccta aactccaatc tacttgtcat ggggttgaaa tcacgatagt taatgtgcta ggtaaggggt

1050
*
atggcgcgtt gcatttagct ttcgatggga ttcgatcgtt ttcccatgac gccttcactc tcgaaaccaa

FIG. 12(II)

1120
*
tgtcacattt tgagatcttg gactttgttt ccaccaaggg attgcgccat gcagctcctc acccgcgtcc

1190
*
ggacggtagc acacgagagg aaccatgaag gcgccttcga catgcgggcc ttggatgggt cgacgaaaaa

1260
*
ggtcctaggt tcgcggccta atgtcgcacg acccgatgct tcgtacaagg tctatagaac ggttagggca

1330
*
taagggcacc tagttcaaaa aactcaaaag ggcccaaccg agggtagggt tggcagcggg cgacgaagcg

1400
*
aatatagccg cacgtgccac cacacaaaat gagggctaat cttcgatgtc gcaccgtcta ggacccatca

1470
*
tgcatgtagg atccatcttc gatgtcaatc acgatccctc atgctcttac gacctcctcg acacgccttc

1540
*
gtgcgattgc tagaggacat tgtcgacgga atatcccctc tttgccctgt gacttggatg attatacatt

1610
*
tatggtaggt gttatggatg ttataaatgg atgtatatga atgtgtgtgt atctatgtgt tgtggatgaa

1680
*
tataaataat tattttctaa ctggtaagaa tcatttctgg tgactaggtt cagtcgataa aaattagtat

1750
*
gtctaatttg tgtattatgt ctatgaaaat tagttaattt tagtttatta atcttcaaaa gttacagacc

1820
*
gacgaaaact agactatcag tcacaactgg taagaaggaa caacgacaac agagatgcca agttactggc

1890
*
ttactgcagc aagctaccgt tttctgcccg cgtgtacatt gaagcacagg tgcgtctaca ctctacgctc

1960
*
tcgagtccaa tataaaaata gactgttggg cacctattgt acccgtaccc ctgttcctgc tcctgccgca

2030
*
gtactgaatt ctgctgctgc tacactcctc tgtccgcatc catccacgtc tctctcctct gccgcccgcc

2100
*
tgcgccaccc atcactgtgc gcgtctcccg catcgtccgc tctctttctt tttcaccctt tcccggccca

FIG. 12(III)

2170
*
tcttctcttt ttacatctgc aacggcaggc cggctgcggc agcggcagcg gcagctgacc agtgaccgac

2240
*
caccccaca ccactccggc gccccaatcc tccccttct tctttttcac tactactact gtactgcacg

2310
*
gtcgccaagc gccagaacgc agtggagaac ggggggcagg actccaacaa gcgttgattt ctgccggcac

2380
*
gcacggcacg ggcacgggca cgggcacggg cgtccccct cactcacgca ccctgcgtct tttccggctg

2450
*
ccgctgctgg ctggctggct ctggctcaca gctacaggct acagtgaccg ccacgcaacc cacactgtct

2520
*
ctgtcctcgt ctccctctcc cctcctagct ctagctggat aggtgggctc tggggaggag gaggagggta

2590
*
gctaggtagt agctgcctat aggcctcggc ccccattcat ggccattacc acgatgtgtc accccaccac

2660
*
accgccctct ccgatgctgc ctccctcatg ataaccctct ccctggtggt tgttctttgc cttgttgccg

2730
*
tgcagcctcc acccccaccc tcctcattaa tcacttgcta gctccctgcc tccctcccgg ctcccgctcc

2800
*
◆
cccttctcgt gcttcgcgcc cccgcagcag ccATGGCGGG GATCGACCTC AACGACACCG TGGAGGAGGA

2870
*
CGAGGAGGAG GCGGAGCCCG GCAACGCCTG CTCCCAGCAG AGCCGGACCA GCTCCGCGGC CACGTTCCCG

2940
*
CCGCCGCCGC CGAACCAGCC GAGGCCGAGC GCCGCGGTGT GCCTCGAGCT GTGGCACGCC TGCGCCGGCC

3010
*
CCGTCGCGCC GCTGCCGAGG AAAGGGAGCG TCGTGGTGTA CCTCCCGCAG GGACACATCG AGCACCTCGG

3080
*
※
CGACGCCGCG GCCGCCGGCG GAGGCGCGCC GCCGCCCGTC GCCCTGCCGC CCCACGTCTT CTGCCGCGTC

3150
*
GTCGACGTCA CTCTCCATgt gcgcgcgccg gttcctactc aatgcgtgcg tgtgtggatt gcccgtgccg

FIG. 12(IV)

3220
*
gtgtgcggct tccactgact ctgtccctct tgcgctcgtt gcagGCGGAC GCGTCCACGG ACGAGGTGTA

3290
*
CGCCCAGCTC GCCCTCGTCG CCGAGAACGA Ggtgcgcgca agccacagtg ctccaccggc attggattcg

3360
*
gcttggtttt ctccttgcgt ccacagagac gagatttggg ctgatttggt gtttcttgtg gcgcttgctt

3430
*
▼
cgtgcagGAT GTCGCGAGGC GGCTGCGCGG ACGGTCGGAG GACGGCAGCG CCGAGGACGG CGACGAAGGG

3500
*
GAAACCGTGA AGCAGCGGTT CTCGCGGATG CCGCACATGT TCTGCAAGAC GCTCACGGCC TCCGACACCA

3570
*
GCACGCACGG CGGCTTCTCC GTGCCACGCC GCGCCGCCGA GGACTGCTTC CCGCCTCTGg tacgcttgcg

3640
*
ttggcttgga aagcttccat cttttgggtg cccgggtgct gctctcaagt gcgattctga atcatctgct

3710
*
cttggggcgt gcagGACTAC AGCCAGCAGC GACCGTCGCA GGAGCTTGTG GCCAAGGATT TGCACGGAAC

3780
*
CGAGTGGAGG TTCCGCCACA TTTATCGAGg tacatgaaca aataatgaga tacaagacga gcacatctac

3850
*
ctatttcttt agcaaactta tgtgcttgct cgccctgaat cattcagtgt cagcgaatga tgtcaatggc

3920
*
tgcacttcag ttggtgattg ttagcgtttt tttacaggat ttgcattact tgtttggatt gagcacttgg

3990
*
gaatgcttca tctttgctca cttaagtcca ggatttgaag tcattgttca gtcactcttt tgctatatat

4060
*
gtcaccatta tgtgatcaga actactaatg gttatatgtt gagagagata tacaaactat gtcaatgttt

4130
*
cctgctgtct gcatttgcaa ccttgtgcgc tatgctcagc atttctcatg tcattggtta gttattgtag

4200
*
tcgtacttaa aatttaccat tttgtccatg aaaaatcatc tgattatatG TTCAGGAGTT CTGGTCCGGT

FIG. 12(V)

4270
*
TTTAAGGAAT GTAAAAGAAC AAACATGAGA AGCTATGTCA TGTGTGGTCC TTGGTTTCTG ATGAATCTGC

4340
*
ATCTGAATGT GATGCAGGGC AGCCCCGCAG ACACCTTTTA ACCACTGGAT GGAGTGCCTT TGTCAACAAG

4410
*
AAGAAGCTTG TCTCAGGGGA CGCCGTACTA TTTTTGAGgt aggccacagc taacattgga gataattatc

4480
*
acatgttggt gttggcccct tctgaagatt cctcataatt ttcagGGGTG ATAATGGGGA GCTAAGACTT

4550
*
GGAGTGCGCC GTGCAGCTCA GCTTAAAAAT GGATCTGCTT TTCCAGCTCT TTATAACCAG TGCTTAAATC

4620
*
TTGGTTCACT ACCTAATGTT GCACATGCTG TGGCCACCAA AAGTGTGTTC CACATCTACT ACAACCCCAG

4690
*
gtgatgatga atatagcggt ttcactttaa tgcttttgca tgttcaattg ttcatgttgt tggcactctt

4760
*
ttagatgatg tgaactgaaa tgtgctatta actatactct ttcaattgac ggcgatttga aattgtgtca

4830
*
ttttgtgtga tatcatttcc tgagttgttt cgaactatgt aattcatgat tcttactgca attcaacatt

4900
*
aagtgatata taattacttt ttgaattgat attgtcactt acatttggac ccttcaatat aatatagttc

4970
*
cacagctctt tttttagata tcatgacaag tacgcaagta gatctttggt tccttatgta tctcatgtgc

5040
*
atttttacct tcttggaccc tgatgtgttg ctgcaagcct tacctttttа tccaccaaca atgatggccc

5110
*
tgatggcaat tattgctttc caaaaatctt acagATTAAG CCAATCTGAA TTCATTATAC CATTTTCGAA

5180
*
GTTTATCAAG AGCTTCAGTC AACCATTTTC TGCTGGTTCG AGGTTCAAAG TGAAATATGA GAGTGATGAT

5250
*
GCTTCTGAAA GAAGgttggt gtgctacagt tctcatcttt tacatagatt tatgatggtt gacacatgag

FIG. 12(VI)

5320
*
agtattatgc agATGCACAG GGATCATAGC AGGAATTGGT GATGCTGACC CCATGTGGCG TGGTTCGAAA

5390
*
TGGAAATGTT TGATGgtatg ttgcctttta agctttaatg attcactttc tgtataactt ttcaggtggt

5460
*
aaatttgtgt tacatatgaa aataatccat gttagataca tgttgaatat aacatgtttc tttatacaga

5530
*
acactaggcg tgtgcatcat gtagctgccg ttgccatcta tttgcactat ttgcttgcta ataaaccaat

5600
*
aagcaatctt gcatatctat ccaataatac aatgcacaac aaatgttgaa aattgcaatt gagagcctac

5670
*
tatgcatccc gtgctccctg agctgtctct gtttgatgta caagtttaat tgtaatgaca catttttttt

5740
*
gcatgtaagt agttctcctt ctccagagca cattctttga tgagcctcat cttagaggca tgttgtatct

5810
*
ttatctaaaa gagactgcct tgtgccagcc tggtttcctt gatcagggct ctaagtaaat aagttcattt

5880
*
cattttggtt tcttattgcc ctgcccctga gtgcacattg taggggtaca taataccctc ttgacttagt

5950
*
aagccagttc taaattgccg caatcttaat cctcttgatg accttacata ttttgtatat aaaccaatgg

6020
*
ttcatttttg cagGTTCGAT GGGATGACGA TGTAGATTTT CGTCAACCAA ACAGGATTTC TCCTTGGGAG

6090
*
ATTGAGCTGA CTAGTTCAGT TTCAGGATCT CACATGTCTG CACCAAATGC AAAGAGACTG AAACCATGTC

6160
*
TTCCCCATGT TAATCCAGAC TACCTAGTTC CAAgtatgcc ctgttctgcc cagatgttcg cttaatgatt

6230
*
attttgttag cttccgtcat gaataatatt ttcattttga tagATGGAAG CGGTCGTCCT GATTTTGCGG

6300
*
AATCTGCCCA ATTCCACAAG GTCTTGCAAG GTCAAGAATT ACTGGGTTAT AGAACTCATG ACAATGCTGC

FIG. 12(VII)

6370
TGTTGCAACT TCTCAGCCAT GCGAAGCAAC GAACATGCAG TACATTGATG AACGAAGTTG CTCCAACGAT

6440
GCGAGTAACA TTATCCCGGG GGTTCCAAGA ATTGGTGTCA GAACACCACT CGGAAGCCCT AGGTTTTCCT

6510
ACCGTTGCTC AGGCTTTGGG GAGTCTCCAA GATTCCAAAA GGTCTTGCAA GGTCAAGAAG TATTTCATCC

6580
CTACAGAGGA ACTCTGGTCG ATGCAAGCTT GAGTAATAGT GGCTTCCATC AGCAAGATGG TTCTCATGTG

6650
CCTACTCAGG CCAGCAAGTG GCACGCACAG CTACATGGAT GTGCTTTTCG TGGCCAACAA GCACCAGCTG

6720
TTCCATCTCA ATCCTCATCC CCACCATCTG TCCTGATGTT TCAACGAGGT GATCCAAAGA TGTCCCCATT

6790
TGAATTTGGG CATTTCCACG TGAATAAGAA AGAGGATAGA CGCGCAATGT TTGTCCATGC TGGAGGCATC

6860
GGAGGAACTG AGCAAACGAC GATGCTCCAG GCTCATCATG TTTCTGGAGG AACGGGAAAC AGAGATGTGA

6930
CCGTTGAGAA ATCTCATCCC GCTGTTGCCG CTGCTTCAGA CAACAGGGAA GTTAGCAAAA ACAGTTGCAA

7000
AATATTTGGC ATATCTTTGA CCGAGAAGGT TCCAGCAATG AAAGAAAAGG GCTGTGGTGA CATCAACACC

7070
AACTATCCAT CCCCCTTCCT GTCTTTGAAG CAACAAGTGC CGAAATCGCT GGGCAACAGC TGTGCCACCg

7140
tgagtgtcct acaccatgta gcacccttga tgtctttctc gagtgaagta actcttaact attataaaat

7210
cctgcacGTT CATGAGCAGA GGCCTGTTGT TGCTAGGGTG ATTGACGTTT CAACAGTGGA TATGATGATC

7280
TGATGTATTG GAAAACTGTC CTGGAGgtga agtcatgcta gtaccacctc tgtcttcatg ctagtgacca

7350
tgaacagcat caaagcattt taagctgact gttcttaagc acatcgctta ttgttgttgc cttgtgtttt

FIG. 12(VIII)

7420
*
tgcagGCTGT GTTGCGTAGT GTGGACAGTG TCGGTTTGAT GGTTCGGTAT CGTGAAGACG GGATTTGATT

7490
*
GAGGATCTGG CCAGATTTGT ATCCTAGTTG TAGCTGTTAG AGCACTTTGT ATGACAACCG TGAGTGCTCC

7560
*
GTGTTATCAG CACTAGTTGC TGCTCACAAC TTGCCTCTAT GTTCATAATC TGTATGCCAT GTCAGACCCA

7630
*
TTTATAGAGG GTTTGTTTGC TTGGCATAGT TCTAGACTTA AAGCATTATT ATGAGAACAA ATTTGCTCTG

7700
*
Caccgtatct ttcttacttt caagttggca acggattaac ggtggaggag atgatctgag aggttagttg

7770
*
tgcgacgtat taatggtgtt acatatatta tgcttaggag cattctgcca gctcatttat catatacatg

7810
*
tcagcacttg atttgttaag tgtagttagt agccttgcac tttgg

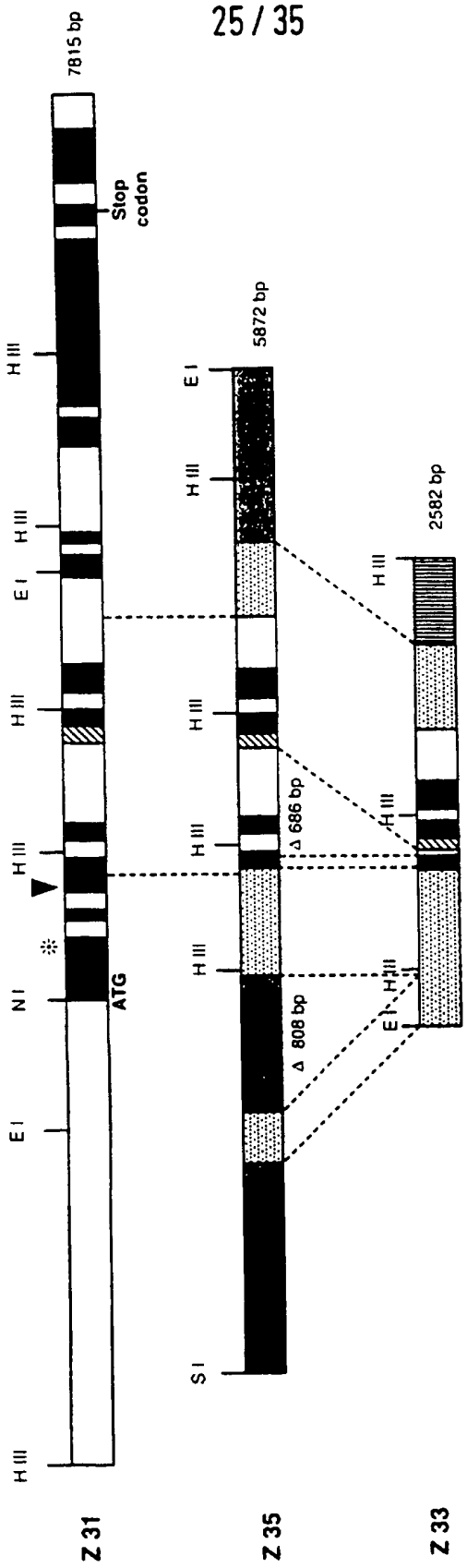

7815 bp
Stop codon
H III
E I
N I
ATG
5872 bp
S I
Δ 686 bp
Δ 808 bp
2582 bp
1000 bp
Z 31
Z 35
Z 33

FIG. 13

FIG. 14

```
Z31       MAGIDLNDTVEEDEEEAEPGNACSQQSRTSSAATFPPPPPNQPRPSAAVC
Zm41-A    ---------------------------------------------------

Z31       LELWHACAGPVAPLPRKGSVVVYLPQGHIEHLGDAAAAGGGAPPPVALPP
Zm41-A    ---------------------------------------------------

Z31       HVFCRVVDVTLHADASTDEVYAQLALVAENEDVARRLRGRSEDGSAEDGD
Zm41-A    ----------------------------------------DGSAEDGD
                                                   ********

Z31       EGETVKQRFSRMPHMFCKTLTASDTSTHGGFSVPRRAAEDCFPPLDYSQQ
Zm41-A    EGETVKQRFSRMPHMFCKTLTASDTSTHGGFSVPRRAAEDCFPPLDYSQQ
          **************************************************

Z31       RPSQELVAKDLHGTEWRFRHIYRGQPRRHLLTTGWSAFVNKKKLVSGDAV
Zm41-A    RPSQELVAKDLHGTEWRFRHIYRGQPRRHLLTTGWSAFVNKKKLVSGDAV
          **************************************************

Z31       LFLRGDNGELRLGVRRAAQLKNGSAFPALYNQCLNLGSLPNVAHAVATKS
Zm41-A    LFLRGDNGELRLGVRRAAQLKNGSAFPALYNQCSNLGSLPNVAHAVATKS
          ********************************** ****************

Z31       VFHIYYNPRLSQSEFIIPFSKFIKSFSQPFSAGSRFKVKYESDDASERRC
Zm41-A    VFHIYYNPRLSQSEFIIPFSKFIKSFSQPFSVGSRFKVRYESDDASERRC
          ********************************** ******.***********

Z31       TGIIAGIGDADPMWRGSKWKCLMVRWDDDVDFRQPNRISPWEIELTSSVS
Zm41-A    TGIIAGIGDADPMWRGSKWKCLMVRWDDDVDFRQPNRISPWEIELTSSVS
          **************************************************

Z31       GSHMSAPNAKRLKPCLPHVNPDYLVPNGSGRPDFAESAQFHKVLQGQELL
Zm41-A    GSHMSAPNAKRLKPCLPHVNPDYLVPNGSGRPDFAESAQFHKVLQGQELL
          **************************************************

Z31       GYRTHDNAAVATSQPCEATNMQYIDERSCSNDASNIIPGVPRIGVRTPLG
Zm41-A    GYRTHDNAAVATSQPCEATNMQYIDERSCSNDASNIIPGVPRIGVRTPLG
          **************************************************

Z31       SPRFSYRCSGFGESPRFQKVLQGQEVFHPYRGTLVDASLSNSGFHQQDGS
Zm41-A    SPRFSYRCSGFGESPRFQKVLQGQEIFHPYRGTLVDASLSNTGFHQQDGS
          ***************************.***************.********

Z31       HVPTQASKWHAQLHGCAFRGQQAPAVPSQSSSPPSVLMFQRGDPKMSPFE
Zm41-A    HVPTQASKWHAQLHGCAFRGPQAPAVPSQSSSPPSVLMFQRGDPKMSPFE
          ********************* ******************************

Z31       FGHFHVNKKEDRRAMFVHAGGIGGTEQTTMLQAHHVSGGTGNRDVTVEKS
Zm41-A    FGHFHVNKKEDRRPMFVHAGGIGGTEQTTMLQAHHVSGGTGNRDVTVEKS
          **************.**************************************

Z31       HPAVAAASDNREVSKNSCKIFGISLTEKVPAMKEKGCGDINTNYPSPFLS
Zm41-A    HPAVATASDNREFSKNSCKIFGISLTEKVPAMKEKGCGDINTNINTNY--
          *****.******* ********************************  . .

Z31       LKQQVPKSLGNSCATVHEQRPVVARVIDVSTVDMMI
Zm41-A    -----PKSLGNSCATVHEQRPVVGRVIDVSTVDMMI
               ******************.*************
```

FIG. 15(I)

70
*
gaattcaagg gagaagatga tttatcagca ggctctatga gcacagctgc aaagtcaaga cataattctt

140
*
gggcctctgc aggtgattct cacccctact ctgacattgc ttgcccttca aaaatattca gtcaagacaa

210
*
aaaagaactt actaatcaaa tgtcattatc agtcaatact ttaagataag tagaatcgat gtcccatacg

280
*
acattctagc cacgcactta aacatgtgcc agatatgttc agatcttgtg attcaacaga cctcgacgcc

350
*
gactttcatg tatatctttt aggttgaagc ttttgcttag ttcagtgttg ctatcagaaa gctaaaatta

420
*
ttttcttgcc acctcctctg catttttac tgcttcagct cctggtgctt ctaatcgagt actatagaaa

490
*
gcatctccct tgataaatcg ttgtgtgcaa atatagggtg cttatataat ccatcattag agtatgaggc

560
*
gtgctttatt ctatgtgctt cccacaaaaa gagtagccta ttataaactt tgtattagag cacatgacgt

630
*
tctaagtttt gaccacattt ctctactatt atattgcagc cataaagatt caatttttat gttgggcacc

700
*
ataaagatgt ttggcaccat tcttcccaaa catttatcta ctattataat gcatgcttta ttcaattttt

770
*
agtattgtta ggggtgaagt cttagtctca agatagcata ttgttgtttg cctactccga cgactctgac

840
*
gaggctgctg ccccgcgcca ggagggaggt caagaagcct aagaagccca agGTGAAGCA ACGATTCTCG

910
*
CGGATGCCGC ACATGTTCTG TAAGACGCTC ACGGCCTCCG ACACCAGCAC ACACGTCGGC TTCTCCGTGT

980
*
CGCGCaagga cagagcaagc tatgtCATGT GAAGCTATGT CATGTGTGGT CCTTGGTTTC TGATGAATAT

1050
*
GCATATGAAT GTGATGCAGG GCAGCCCCGC AGACACCTTT TAACCACTGG ATGGAGTGCC TTTGTCAACA

FIG. 15(II)

1120
*
AGAAGAAGCT TGTCTCAAGG GACGCCGTAC TATTTTTGAG gtaggccaca actaacattg gagataatta

1190
*
tcacatgttg gtgttggccc tttctgaagg ttcctcataa ttttcagGGG TGATAATGGG GAGCTAAGAC

1260
*
TTGGAGTGCG CCGTGCAGCT CAGCTTAAAA ATGGATCTGC TTTTCCAGCT CTTTATAACC AGTGCTCAAA

1330
*
TCTTGGTTCA CTACCTAATG TTGCACATGC TGTGGCCACC AAAAGTGTGT TCCACATCTA CTACAACCCT

1400
*
AGgtgatgat gaatatagcg gtttcacttt aatgtttttg catgttcaat tgttcatgtg gttggcactc

1470
*
ttttagatga tgtgaattga aatgtgctta ttaactactc tttcaattga cggggaattt gaaattgtgt

1540
*
cattgtgtgt gatatcattt cctgagttgt ttcgagctat gtaattcatg attcttactg caattcaaca

1610
*
ttaagtgata tataattact ttttgaattg atattgtcac ttacatttgg acccttcaat ataaatcttt

1680
*
ccaattaatg ctctttttat ccactctttg ttgtcaagtt tctgcaattt agaagtatgc tttcttttgt

1750
*
atttaattct ttttaggcca cagattgtta tttcttcatg ccataatttc tctgttttat tagtcatagt

1820
*
aacagaaata tttttcaatt gttgtggcgg ctggccttga ctgctatggc ggtggccgga ctggccagcg

1890
*
atggcggtgg ccggatagca ccgcgagagc aacgtccaga ggctagcagt tcgttggttg ttgagatttg

1960
*
taccaatgat tatctatatt tagagttgtt gttggataca cccatccatt tagtccttgt ctatctttta

2030
*
cacaaccatc taaactataa atttagctag gattataaat aagctgttgg agttgctctt aggtggctcc

2100
*
tccaatatag gattagtcca tttttctaca aactttgatg tgaattgagt ttctgccaat catgttatat

```
                                                                    2170
                                                                     *
atgcatatgt gatgtgaatt gagattcatt gagcaacaca aggattctgt gttggagatg gggtcttaat
                                                                    2240
                                                                     *
atttctatca tgtaatatct tttggtagct tgcatcatat taataaaata tctttggtgg cctcaggtct
                                                                    2310
                                                                     *
ggtggtaatg cttatgtgat tggtgattct gcaaagcctg agcagaagtg gcacgcctac tatgccacta
                                                                    2380
                                                                     *
ctgagcaccc ctgaggagct tgttgttact cttaacatgt gcatgactgg gctggacaag aagagagctt
                                                                    2450
                                                                     *
ctgtcttctt ctaggcttct gctgatggtt acacatcttg tgctaaggag atgaccaagc tctcaggtat
                                                                    2520
                                                                     *
ctcggacatt atcctataga cagagatctg cgactaattt gttaggttgg ttcttcatca ttttgtagat
                                                                2580
                                                                 *
gcccttcctt ctcgctacat gaactaacta atgacagagg gtggaagtga cccatgaagc tt
```

FIG. 15(III)

FIG. 16(I)

70
*
gtcgacctgc aggtcaacgg atctattgaa ccagcagtct ttgcaattga gatttgactg ccggatttgg

140
*
tttcagcatg gatgcaccac cccacatcat gtggttctag agcatatagt ggtcttgtag cgcctaaaag

210
*
ttttagtagc atcaaatgtc agaaatatat cttcatctcc agaaaatatt agtacttcat aggatgaaaa

280
*
ttgttcaacc tgaaataatt tatttcttgc atccttcagg ttgtatgcga aaccactaga ttgaataatt

350
*
caagaaatct acagaggcag tcgtgaacaa ctatatatgc gcaagattga gcctaaggtt tgtagaccct

420
*
ttaattcata caagggcatt gccatttccc ccgtaatttc gatgcagctc ctttagccat ataacaatga

490
*
aaaccaacga tcctgcaatc ctgaaagggt gaatttatgg gagaagcgta caactccttt agccaatgat

560
*
tccaatgaag caccagccta caagaataag atagataaat taacagggta taaaaatgat actaatcaca

630
*
tgtagtaaaa gaaacttaat ccttccactg catcacgtat atgtgagtgc tccctggttt ttcattacag

700
*
tcttgtgatt tccattttat gctcgatgta ggtataggca tctgatggag gacgttttgt ctctactccc

770
*
gcatgtgaag aaggacaacc aggacaaggt cgagtccaag cagagcaagg ggaacacgct gaacaagttg

840
*
cttgagttca ggagctgctt cagctgcctt tcttcgaggt atagatattc tactgtgcct ccacacagct

910
*
ggtggaaatt ttgttatcat agatacgatg gcggctgctt acatgtggga atcttacact gtataagtca

980
*
gtggcgcaaa tcaaatctcc aacttgggtt tggtccacct ttcgtgaaat gaatgttttc tgggctttca

1050
*
ggtattgagt aaggagctcc cattttgctc tggtgccaaa ttctctacta ggcaattgac gtttttactg

FIG. 16(II)

1120
*
catttgtgac atctgccttc ccacaattat aattgttcaa tatatgtatg cattagactt atcaatttta

1190
*
ttaacttatt gaattgtatg tgcatgaagt tttttctttc atgtattaca ccacatgaca tagttcttta

1260
*
actaatggca gtgtaccttt tttaaccttt agatggctaa attcaaggga gaagatgatt tattagcagg

1330
*
ctctatgagc acagctgcac agtcaagaca taattcttgg gcctctgcag gtgattctca cccctacgct

1400
*
gacattgctt ggccttcaaa aatattcagt caagacaaaa agaacttact aatcaaatgt cattatcagt

1470
*
caatacttta agataagtag aatcgatgtc ccatacgaca ttctagccac gcacttaaac atgtgccaga

1540
*
tatgttcaga tcttgtgatt cagcagacct tgacgccgag cgggcctccg cggaggcagt agccagatct

1610
*
ggccattgag tgccccgacg ccgctgctta ctcatccatc gccgcggtga cctgctcccc ctcgggcata

1680
*
tctgtccatt gacaccaagc atgttctttc ctgaactgtt ctaaaagttc agtttcatgg ttgtttattc

1750
*
ttttgatcag gaaggagaga aagggagaat cagttagaag aaagaagagt ctgaaagctg agtaatttac

1820
*
ctcaacttta ctacccatgt tattaagatc tattgatgat cgtcccactt actcctatga tgcacagact

1890
*
taatggatca tggactgaca tatttatcac gggttttggg ttgtcttcct tcccagtttt gttttaccag

1960
*
tggagacacg aagattggag gacataaggg cgcaacacag gactacagcg agggggaagg ccagatcaag

2030
*
caggagacaa caagaggtgg gttgctgctc attcacaatt tgatatgttt gttttttcgt tgttatagct

2100
*
gaactgcaca tgcagtttga aacatgttgt tactgatgtg tttgtctatt acaggatgtg atagatggtg

FIG. 16(III)

2170
*
atctctgtga gcagtatccc tccctcctag ctgatatgca gaggaagatt gctgatgagc tggacagaag

2240
*
tccgacgcct gcagcactgc ttggtgagga ttgccaagga ggaagactag aacaagcaag agcagcgtta

2310
*
atcagtgaca gagcatgatg ccatccagat gggacaagat aagtaagcag tcttatatag tctgcccact

2380
*
cgagttttgt atatatttta ggttgaagct tttgcttagt tcagtgttgc tatcggaaag ctaaaattat

2450
*
tttcttgcca cctcctctgc attgttttgc tgcttcagct cctggtgctt ctaatcgagt actatagaaa

2520
*
gcatctctct tgataaatcg ttgtgtgcaa atatagggtg cttatataat ccatcattag agtatgaggc

2590
*
gtgttttatt ctgtgtgctt cccacaaaaa agagtagcct attataaact ttgtattaga gcacatgacg

2660
*
ttctaagttt tgaccacatt tctctactat tataatgcag ccataaagat tcaattttta tgttgggcac

2730
*
cataaagatg tttggcacca ttcttcccaa acatttatct actattataa tgtgtgcttt attcaatttt

2800
*
tagtattgtt aggggtgaag tcttagtctc aagatagcat attgttgttt gcctactccg acgactctga

2870
*
cgaggctgct gccccgcgcc aggagggagg tcaagaagcc taagaagccc aaggtgaaga agcccaagGT

2940
*
GAAGCAACGA TTCTCGTGGA TGCCGCACAT GTTCTGCAAG ACGCTCATGG CCTCCGACAC CAGCATGCAC

3010
*
GTCGGCTTCT CTGTGCTGNG CCGCTCCGCC GAGGACTGCT TCCCGCCTCT Agtacgcttg cgttggnttg

3080
*
gaaagcttcc atcttttcgg tgcccgggtg ctgctctcaa ggtgtgattc tgaatcatct gctcttgggg

3150
*
cgtgcagGAC TACAGCCAGC AGCGATCGTC GCAGGAGCTT GTGGCCAAGG ATTTGCACGG AACCGAGTGG

FIG. 16(IV)

3220
*
AGGTTCCGCC ACATTTATCG AGgtacatga acaaatactg agatacaagc cgagcacatc tacctatttc

3290
*
tttagcaaac ttatgtgctt gctcgccctg aatcattcag tgtcagcgaa tgatgtcaat ggctgcactt

3360
*
cagttgatga ctgttagcgc tttttacagg atttgcatta cttgtttgga ttgagcactt aggaatgctt

3430
*
catctttgct cacttaagtc caggatttga agtcattgtt cagccactct tttgctatat atgtcaccat

3500
*
tatgtgatca gaactaataa tggttatatg tcgagagaga tatacaaact atgtcaatgt ttcctgttgt

3570
*
ctgcatttgc agccttgtgc gctatgctca gcatttctca tgtcattggt tagttattgt agttgtactt

3640
*
aaaaattacc attttgtcca tgaaaaatca tctgattata tgtTCAGGAG TTCTGGTCCC GTTTAAAGGA

3710
*
ATGTAAAAGA ACAAACATGA GAAGCTATGT CATGTGTGGT CCTTGGTTTC TGATGAATAT GCATCTGAAT

3780
*
GTGATGCAGG GCAGCCCCAC AGACACCTTT TAACCACTGG ATGGAGTGCC TTTGTCAACA AGAAGCTTGT

3850
*
CTCAAGGGAC GCCGTACTAT TTTTGAGgta ggccacaact aacattggag ataattatca catgttggtg

3920
*
ttggcccttt ctgaaggttc ctcgtaattt tcagGGGTGA TAATGGGGAG CTAAGACTTG GAGTGCGCCG

3990
*
TGCAGCTCAG CTTAAAAATG GATCTGCTTT TCCAGCTCTT TATAACCAGT GCTCAAATCT TGGTTCACTA

4060
*
CCTAATGTTG CACATGCTGT GGCCACCAAA AGTGTGTTCC ACATCTACTA CAACCCCAGg tgatgatgaa

4130
*
tatagcggtt tcactttaat gcttttgcat gttcaattgt tcatgttgtt ggcactcttt tagatgatgt

4200
*
gaactgaaat gtgcttatta actactcttt caattgacgg ggatttgaaa ttgtgtcatt gtgtgtgata

FIG. 16(V)

4270
*
tcatttcctg agttgtttcg agctatgtaa ttcatgattc ttactgcaat tcaacattaa gtgatatata

4340
*
attacttttt gaattgatat tgtcacttac atttggaccc ttcaatataa atctttccaa ttattgctct

4410
*
ttttatccac tctttgttgt caagtttctg caatttagaa gtatgctttc ttttgtattt aattcttttt

4480
*
aggccacaaa ttgttatttc ttcatgccat aatttctctg ttttattagt catagtaaca gaaatatttt

4550
*
tcaattgttg tggcggctag ccttgactgc tatggcggtg gccggactgg cctgagatgg cggtggccgg

4620
*
atagcaccgc gagagcaacg tccagaggct agcagttcat tggttgttga gatttgtacc aatgattatc

4690
*
tatatttaga gttgttgttg gatacaccca tccatttagt ccttgtttat cttttacaca gccatctaaa

4760
*
ctctaaattt agctaggatt ataaataagc tgttggatgc tcttaggtgg ctcctccaat ataggattag

4830
*
tccatttttc tacagatggg gtgatagcat gcacattcta gcatacacat gcccttggcc tggtaatgct

4900
*
tggatttttt tctcacgcaa aagaatatac cggttcgttg aattatgtga tgtcattttc tacttttctg

4970
*
ttttttagcc gatcatccga aggctaatga atattaccct gacccaagat tagtagcata tgttgtaccc

5040
*
tatgcaccta tcctatcgtg gtatcactaa tccttctaaa tttgatatca tcttatctga ttcagcttgt

5110
*
tacttgattt aatttggctc cttgttaaca gtacggatgc tgcaaaaaat tccctgagga gaaaggttga

5180
*
aatcttaaaa ttgaagcctc attggtccaa agcttacttc tatttgtggg atgaggtgcg ttattttacc

5250
*
ttttctgcta tgtcctgatt tcaggggaca ccagtgcaga tgcatgtagg gagaaacttg ttgcagttac

5320
*
agaaatggtt tccaatatct actcttgcaa ttgaagatat ggagttactc cttgggttct ccttttagtt

5390
*
ttattatgct cgtccagtag acatgctcct gtagtaaact tatattcatg cttgtaattc catttacaat

5460
*
gtgaatattg tgtatagtag ccatgacatg ataatagatt gttagggtca ctcatcaaat attactatgt

5530
*
gccgtcacaa atatgggcac tccactaggg tttagggttt tacctgttgt gcccagttag ggtcactcat

5600
*
caaatattac agagggtatg ttccatttac agttggagta gatacgcatg acgggggcgc acatgagtta

5670
*
ttagtcttgt cgggatctca tgagtctgat tgacgtattt cggatggctc tcgacgtgcg ggtcgacgac

5740
*
ggaacacttg cagcgcccat gttcggatgc agcgacagcc tccttgtgtc ttcgaactcg cgacgagaga

5810
*
gagtggtatt caggactgct tgcttacagg agagaaataa gctaatttct cagaatctta gaagctgatt

5870
*
ttacaacagg attgcttgct tacagagttg atcaactaaa aaagcgctat ggttcagaat tc

FIG. 16(VI)

INTERNATIONAL SEARCH REPORT

Ir ·ational Application No
PCT/GB 96/03191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 C12N15/11 C12N5/10 C07K14/415
A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL Heidelberg, BRD AC D40316, 13 November 1994 SASAKI T. ET AL.: "Rice cDNA from shoot" XP002031170 see abstract & UNPUBLISHED, SASAKI T. ET AL.: --- -/--	1,6, 21-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

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'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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'&' document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 1997	30. 05. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

[r ·ational Application No
PCT/GB 96/03191

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL Heidelberg, BRD AC Z34707, 25 June 1994 PARMENTIER Y. ET AL.: "The Arabidopsis thaliana transcribed genome: the GDR cDNA program" XP002031171 98 % identity to bp 98-1 (antisense) of Ms41-A see abstract & UNPUBLISHED, PARMENTIER Y. ET AL.:	15,21-23
A	TIBTECH, vol. 13, September 1995, pages 344-349, XP002031169 WILLIAMS M.: "Genetic engineering for pollination control" see the whole document	1-29
A	WO 92 13957 A (PLANT GENETIC SYSTEMS NV) 20 August 1992 see the whole document	1-29
A	WO 94 25593 A (CT VOOR PLANTENVEREDELINGS EN ;STIEKEMA WILLEM JOHANNES (NL); PERE) 10 November 1994 see the whole document	1-29

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/03191

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2103572 A	08-08-92
		EP 0570422 A	24-11-93
		US 5589610 A	31-12-96
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		EP 0698098 A	28-02-96